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PHLPP: A Novel Family of Phosphatases that are Critical Regulators of Intracellular Signaling Pathways

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The dissertation of John F. Brognard Jr. is approved and it is acceptable in quality and form for publication on microfilm:

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Dedicated to Karen Brognard for her loving support
and dedication during the many long hours spent
pursuing research and preparing this dissertation.

Dedicated to John and Donna Brognard for their
never ending support throughout my life
and career. Without their support and guidance
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"Pursue your passion" – John F. Brognard Sr.

Dedicated to Dr. Alexandra Newton for her unfaltering optimism
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The text of chapter 2 is, in full, a reprint of the material as it appears in Molecular Cell, 2007, Brognard J, Sierecki E, Gao T, Newton AC. I was the primary investigator and author of this research paper.

The text of Chapter 3 is, in full, a research article to be submitted to Cancer Research December 2007, by Brognard J, Niederst M, Braughton L, Reyes G, Titus-Ernstoff L, Newton AC, Egan KM. I was the primary author and researcher. Niederst M, Braughton L and Reyes G provided technical support. Titus-Ernstoff L and Egan KM provided samples for sequencing. Newton AC supervised the research.

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ABSTRACT OF THE DISSERTATION

PHLPP: A Novel Family of Phosphatases that are Critical Regulators of Intracellular Signaling Pathways

by

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Doctor of Philosophy in Biomedical Sciences

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A fundamental aim in cell signaling is to decipher how phosphorylation and dephosphorylation regulate cell fate. In order to achieve this goal the scientific community must have a complete understanding of the proteins involved in regulating cellular processes. To this end we have discovered a novel family of phosphatases referred to as PHLPP, (PH domain leucine-rich repeat protein phosphatase). These Ser/Thr specific phosphatases have emerged as important regulators of intracellular signaling pathways by acting as the brakes to lipid second messenger signaling. This

thesis will focus on characterizing the novel phosphatase PHLPP2 and defining its role in regulation of the PI3K/Akt pathway. Second, it addresses whether these phosphatases are involved in the process of tumorigenesis. Lastly, it will examine the role of PHLPP in regulation of the related kinase, protein kinase C. In summary, this thesis is a first step in understanding the role of PHLPP in normal tissue homeostasis as well as pathophysiological states.

Chapter 1

An Introduction to the Ser/Thr Phosphatase PHLPP

Abstract

Phosphorylation and dephosphorylation control the biological function of Akt, a key pro-survival kinase. In an effort to understand how Akt signaling is terminated, our lab has discovered a novel family of phosphatases referred to as PHLPP for PH domain and Leucine rich repeat Protein Phosphatase. By screening the NCBI database for a protein containing both a PH domain and a phosphatase domain, we identified the first family member, PHLPP1. We hypothesized that this phosphatase could be localized to the membrane and potentially dephosphorylate Akt (also known as PKB). Biochemical analysis revealed that PHLPP1 does directly dephosphorylate the kinase at a critical phosphorylation site, Ser 473, referred to as the hydrophobic motif. Scanning the NCBI database for other PHLPP homologs revealed a second gene encoding a novel PHLPP isoform, which we have named PHLPP2. This second PHLPP isoform shares 55% amino acid identity with PHLPP1. Understanding why mammals have two genes encoding two unique isoforms of the PHLPP phosphatase will be a major focus of this thesis research. This chapter aims to provide a detailed background of the PHLPP phosphatases and a historical perspective of what was known about PHLPP1 and Akt signaling at the time the research began. It will provide a framework to understand the important questions that have been addressed by this thesis.

PHLPPs: a novel PP2C-type phosphatase family

The PHLPP family of phosphatases is comprised of three members: PHLPP1α (1205 amino acids), PHLPP1β (1717 amino acids), and PHLPP2 (1323 amino acids) (1, 2) (Figure 1A). PHLPP1α and PHLPP1β are splice variants from the same gene, located at chromosome 18q21.33 (1). These two isoforms are generated from two unique transcripts, rather than generated from the same transcript having alternative initiation of translation start sites. PHLPP1β varies from PHLPP1α by an N-terminal extension that includes a Ras association (RA) domain, but otherwise these two isoforms are identical, Figure 1A. The PHLPP2 gene is located at chromosome 16q22.3 (1). To clarify nomenclature present in this thesis, 'PHLPP1' refers to PHLPP1α and PHLPP1β and 'PHLPP' refers to all three family members. PHLPP1β and PHLPP2 possess an identical domain structure with a RA domain, PH domain, leucine-rich repeats, and a PP2C phosphatase domain (Figure 1). PHLPP1β and PHLPP2 share 55% overall amino acid identity with 58% and 63% identity in the PP2C domain and PH domain, respectively (1, 2). It is interesting to note that both genes reside at chromosomal locations frequently lost in various cancers. The region that includes the PHLPP1 gene is lost in a high percentage of colon cancers (3). LOH (loss of heterozygosity) has been observed at the PHLPP2 locus in breast and ovarian cancers, Wilms tumors, prostate cancer, and hepatocellular carcinomas (4-8). Additionally, the PHLPP2 gene spans a chromosomal region that includes a fragile

site (FRA16B). These observations suggest that both PHLPP1 and PHLPP2 could be candidate tumor suppressor genes.

There are three main families of Ser/Thr phosphatases: PPP, PPM, and FCP (9). PHLPP is a member of the PPM family of phosphatases which require Mg^{+2} or Mn^{+2} for their catalytic activity and are not inhibited by traditional phosphatase inhibitors such as okadaic acid (1, 2). The PPM family of phosphatases includes PP2C phosphatases, such as PP2Cα and PP2Cβ, and the pyruvate dehydrogenase phosphatase (10). Phylogenetic analysis using full-length proteins illustrates that PHLPP1 and PHLPP2 constitute a unique subfamily within the PP2C phosphatase family (Figure 1B). The catalytic activity of PPM type phosphatases requires binding of two Mg^{+2} or Mn^{+2} ions, which causes an associated water molecule to act as a nucleophile that can hydrolyze the phosphomonoester bond of a substrate phosphorylated on Ser or Thr residues (9). The PHLPP PP2C domain contains the four invariant aspartic acid residues required for binding to these divalent cations (Table 2, invariant aspartic acid residues are underlined) (10). Most PP2C family members are monomeric, although some do possess other functional domains, notably PP2Cγ, which has a collagen-binding domain, and pyruvate dehydrogenase phosphatase which has a Ca^{+2} binding domain (9). PHLPP is the only PPM phosphatase that contains multiple structural domains involved in cellular signaling, such as the PH domain and the RA domain.

Evolutionary Conservation of PHLPP

Sequence homology analysis reveals that PHLPP is an evolutionarily conserved phosphatase with a homologue in yeast, CYR1 (NCBI, homologene database). The yeast homologue has a RA domain, leucine-rich repeats, and a PP2C phosphatase domain. Interestingly, the C-terminus of CYR1 has an adenylate cyclase (11) domain that is present only in the Ascomycota phylum (which includes Saccharomyces cerevisiae, Schizosaccharomyces pombe, Neurospora crassa). CYR1 is an essential gene in yeast and this attributed to the adenylate cyclase domain (12), however the contribution of the phosphatase domain to this phenotype has not been determined. The PH domain of PHLPP was an addition that occurred later in evolution, and is present in amniotes, which are a class of tetrapod vertebrates that include mammals. The PDZ binding motif is present in Bilateria phylum (animals having a bilateral symmetry). PHLPP1 and PHLPP2 branched off in the Euteleostomi clade that includes 90% of living vertebrates.

Discovery of PHLPP

PHLPP1β, also known as SCOP (suprachiasmatic nucleus (SCN) circadian oscillatory protein), was originally cloned by Shimizu et al, from both the rat and human (13). They discovered PHLPP1β by screening for genes that are differentially expressed in the suprachiasmatic nucleus during the day and night cycles. Interestingly, the human PHLPP1β Shimizu et al cloned lacked 116 amino acids at the N-terminus (13). The complete full length human PHLPP1β has since been cloned by the Newton lab (Matthew Niederst, unpublished data). Gao et al cloned a smaller

splice variant, now referred to as PHLPP1α. The final PHLPP isoform, PHLPP2, was discovered by screening the NCBI database for a PHLPP1 homologue and this isoform has successfully been cloned by Brognard et al (1). This is the only other protein in the database with the domain structure of PHLPP.

Domain Structure of the PHLPP Phosphatase

PHLPP has a unique domain structure with multiple domains associated with signaling. Three domains that have limited characterization are the RA domain, the leucine-rich repeats and the PH domain. At the very N-terminus of PHLPP1β and PHLPP2 is a Ras association domain. It has not been determined if this domain is functional and participates in binding to Ras, but PHLPP1β was reported to bind to both K-Ras (high affinity) and H-Ras (weak affinity) in lipid rafts. This interaction required the leucine-rich repeats of PHLPP1 (14). The leucine-rich repeats are found between the PH domain and the PP2C domain (Figure 1). The PH domain is present in all PHLPP isoforms. Loss of the PH domain does not alter PHLPP1α's ability to regulate Akt phosphorylation or induce apoptosis (2). The PH domain of both PHLPP1 and PHLPP2 contains only the middle Arg in the R-X-R-X-F motif required to bind phosphoinositides, suggesting that if PHLPP does bind phosphoinositides, it does so with weak affinity.

The PHLPP1 phosphatase domain is catalytically functional towards Akt (2). The PP2C domain of PHLPP1 has a catalytic rate of 1 dephosphorylation per second. An identical rate was observed for PP2Cα against the synthetic substrate pNPP (para-

nitrophenylphosphate). The PHLPP1 PP2C domain requires Mn^{+2} for its in vitro catalytic activity towards Akt, a hallmark of PP2C type phosphatases.

Lastly, the PHLPP phosphatases contain type I PDZ binding motifs at their C-termini (15). The motifs for PHLPP1 and PHLPP2 are DTPL and DTAL, respectively. The PDZ binding motif in PHLPP1α is critical to the regulation of Akt hydrophobic motif dephosphorylation, regulation of apoptosis, and tumor suppressor function (2). Expression of a PHLPP1α construct lacking the PDZ binding motif impairs its ability to dephosphorylate Akt (2). The observation that deletion of the PDZ binding motif impairs PHLPPs ability to regulate apoptosis and suppress tumors, suggests Akt is a critical substrate for these functions. How the PDZ binding motif dictates regulation of Akt is the subject of future studies. It is likely that proteins that contain PDZ binding domains contribute to the localization of PHLPP and regulation of downstream substrates, by directing the PHLPP phosphatases to specific signaling complexes within the cell. There is precedent for a PDZ ligand controlling Akt signaling: the PDZ binding motif of the lipid phosphatase PTEN is required to fully suppress the activation of Akt (16).

Targets of the PHLPP Phosphatase

Akt. There are three isoforms of Akt (Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ)) in mammals, and all three isoforms require phosphorylation at the activation loop (Thr 308) and hydrophobic motif to attain full catalytic activity (16). Activation of Akt is dependent upon PI3K generating the lipid second messenger,

phosphatidylinositol 3,4,5 trisphosphate (PIP3), which provides a docking site for proteins that contain PH domains, such as Akt (17). Growth factor stimulation of tyrosine kinase receptors results in activation of PI3K and production of the lipid second messenger PIP3 (18). PIP3 binds to the PH domain of Akt, resulting in a PH-out conformation so that constitutively bound PDK-1 can phosphorylate Akt at the activation loop Thr 308 (19). This is accompanied by phosphorylation of Akt at the hydrophobic motif, (Ser 473 in Akt1), by the kinase complex mTORC2, composed of rictor, mLST8, mSin1 variants, and mTOR (20-23). It is unknown how mTORC2 is activated by growth factors to promote phosphorylation of Akt at Ser 473. Akt signaling is terminated by removal of the lipid second messenger, PIP3, by the lipid phosphatase PTEN (24) and by dephosphorylation of activated Akt.

The first identified substrate of the PHLPP phosphatase was the kinase Akt. PHLPP was first identified by T. Gao et al by screening the NCBI database for a phosphatase containing a PH domain, hypothesizing that this domain could localize a phosphatase to activated Akt, poising the enzyme to be a direct regulator of the kinase. PHLPP does indeed regulate Akt phosphorylation at the hydrophobic motif (Ser 473), but as mentioned above the PH domain is not required for this regulation, thus the serendipitous discovery of Akt as a substrate of PHLPP. The PP2C phosphatase domain of PHLPP1 dephosphorylates the hydrophobic motif of Akt in vitro (2). Consistent with this, expression of full-length HA-PHLPP1α in cells results in a decrease in Akt phosphorylation specifically at Ser 473 (2). Lastly, depletion of endogenous PHLPP1 from cells results in a robust increase in Ser 473 phosphorylation

(2). These data demonstrate that PHLPP1 is a functional phosphatase that regulates Akt phosphorylation at the hydrophobic motif. The PHLPP mediated dephosphorylation of Akt is an evolutionarily conserved pathway, as depletion of PHLPP from drosophila S2 cells causes a robust increase in Akt phosphorylation at the hydrophobic motif (Ser 505 in Drosophila) (2). Highlighting the importance of phosphorylation at Ser 473, Akt singularly phosphorylated at Thr 308 is only 10% as catalytically active as Akt phosphorylated at both Ser 473 and Thr 308 (21, 26). Consistent with these previous reports overexpression of PHLPP1α, resulting in a decrease in Akt phosphorylation at Ser 473, coincided with a decrease in phosphorylation of the Akt downstream substrate GSK-3α/β in cells (2). The decrease in hydrophobic motif phosphorylation also correlated with a decrease in Akt kinase activity in an in vitro kinase assay (2).

Additional characterization of Akt hydrophobic motif phosphorylation revealed that this site is less sensitive to okadaic acid treatment in agreement with a PP2C-type phosphatase regulating phosphorylation at this site (2). Phosphorylation at the activation loop was very sensitive to okadaic acid treatment, consistent with previous reports that PP2A controls phosphorylation at this site (27). The increase in Ser 473 phosphorylation observed in cells treated with okadaic acid suggests other phosphatases also contribute to the regulation of Ser 473 phosphorylation. To ascertain if PHLPP1 is acting directly on Akt in cells, Gao et al performed an eloquent set of experiments using the PI3K inhibitor LY294002. First, they demonstrated that depletion of endogenous PHLPP1 from cells increases Akt phosphorylation at the

hydrophobic motif. They then showed that depletion of PHLPP1 from cells followed by treatment with the PI3K inhibitor still causes an increase in Akt phosphorylation at the hydrophobic motif, and this indicates that PHLPP is not acting at the level of PI3K or upstream, but rather directly at level of Akt. Comparing the rate of dephosphorylation of Akt in cells depleted of PHLPP and exposed to the PI3K inhibitor revealed that Ser 473 phosphorylation was prolonged, while activation loop phosphorylation (Thr 308) was not altered (2). These data convincingly demonstrate that PHLPP1 is regulating phosphorylation explicitly at the hydrophobic motif.

To ascertain the functional consequences of decreased Akt phosphorylation and activity, cell survival was monitored in cells overexpressing PHLPP1α. Overexpression of PHLPP1α resulted in a significant increase in sub-2n DNA, indicative of an increase in apoptosis (2). Importantly, apoptosis can be rescued by phosphomimetic mutant of Akt, S473D, resistant to regulation by PHLPP. PHLPP-induced apoptosis was not rescued by a S473A Akt construct (2). These data indicate that Akt is the main target of PHLPP driving the apoptotic phenotype, and that regulation of phosphorylation at Ser 473 is critical for the observed induction of apoptosis. To further assess the role of PHLPP in regulating Akt-mediated apoptosis cells were depleted of PHLPP1 and treated with the PI3K inhibitor. Consistent with previous results, PI3K inhibition alone results in an increase in apoptosis, which is dependent upon loss of Akt activation (28). Depletion of PHLPP1 from cells treated with the PI3K inhibitor rescued most of the apoptosis induced by this small molecule

inhibitor (2). These results highlight the importance of Ser 473 phosphorylation in regulating cellular survival.

Other Signaling Pathways Regulated by PHLPP. Rat PHLPP1β (SCOP) negatively regulates the Ras-Raf-MEK-ERK pathway by interacting directly with Ras (14). Binding of PHLPP to K-Ras suppresses activation of the Raf-MEK-ERK pathway and results in decreased ERK phosphorylation. The proposed mechanism is that PHLPP binds to GTP free K-Ras, thereby preventing K-Ras from being activated through GTP loading (14). However, other mechanism could explain how overexpression of PHLPP1β suppresses activation of this pathway. Specifically, PHLPP1β could be acting directly on MEK or ERK to dephosphorylate either of these enzymes directly. Both PHLPP1 and PHLPP2 contain putative ERK D domains suggesting that both phosphatases could potentially interact directly with ERK (determined by scansite (scansite.mit.edu)). However it is unlikely that PHLPP directly dephosphorylates and inactivates ERK since this would require dephosphorylation on both Tyr and Thr residues.

PHLPP and Cancer

PHLPP1 is poised to be a potential tumor suppressor based on its chromosomal location, 18q21.33 (a region frequently lost in colon cancers), and its negative regulation of the oncogene Akt (29). Constitutive activation of Akt plays a causal role in cancer by promoting cellular survival and proliferation, hallmarks of tumorigenesis (29, 30). Inhibition of this pathway promotes apoptosis and results in the killing of

cancer cells (28). Consistent with PHLPP1 inhibiting Akt activity, expression of PHLPP1α induces apoptosis in both lung and breast cancer cell lines (2). Similarly, loss of PHLPP1 promotes survival suggesting loss of this phosphatase could tip the balance towards cell survival. Stable expression of PHLPP1α in glioblastoma cells reduces total cell numbers, and inhibits tumor-forming capabilities of these cells when transplanted into mice (2). These experiments strongly suggest that PHLPP1 could be one of the tumor suppressor genes located at chromosome 18q21.

Regulation of the Ras-Raf-MEK-ERK pathway implicates PHLPP1 to be a potential tumor suppressor as well. Activation of this pathway has been documented to play a causal role in many tumors, and inhibitors of this pathway cause cytostatic tumor responses (31, 32). Loss of PHLPP1 may result in constitutive activation of this pathway promoting increased proliferation and contribute to the tumorigenic phenotype.

PHLPP2, what does it do?

Many important questions remain regarding the PHLPP family of phosphatases and their importance in regulation of biological outputs such as cellular survival, proliferation, and cell growth. At the forefront of these questions is determining the role of PHLPP2 in mammalian cells. Why do mammalian cells have two main isoforms of this protein? Does the variability in the sequences of PHLPP1 and PHLPP2 dictate regulation of unique functions and substrates for these two phosphatases? Is PHLPP2 a critical regulator of Akt signaling, and if so, are there

differences in the way these two phosphatases regulate the PI3K/Akt pathway? What will be the repertoire of biological functions these two phosphatases regulate? What other substrates do the PHLPP phosphatases dephosphorylate? It is possible they will regulate phosphorylation of other AGC kinase family members at the conserved hydrophobic motif? Lastly, what role will these phosphatases play in cancer? These important and exciting questions will be the focus of this thesis.

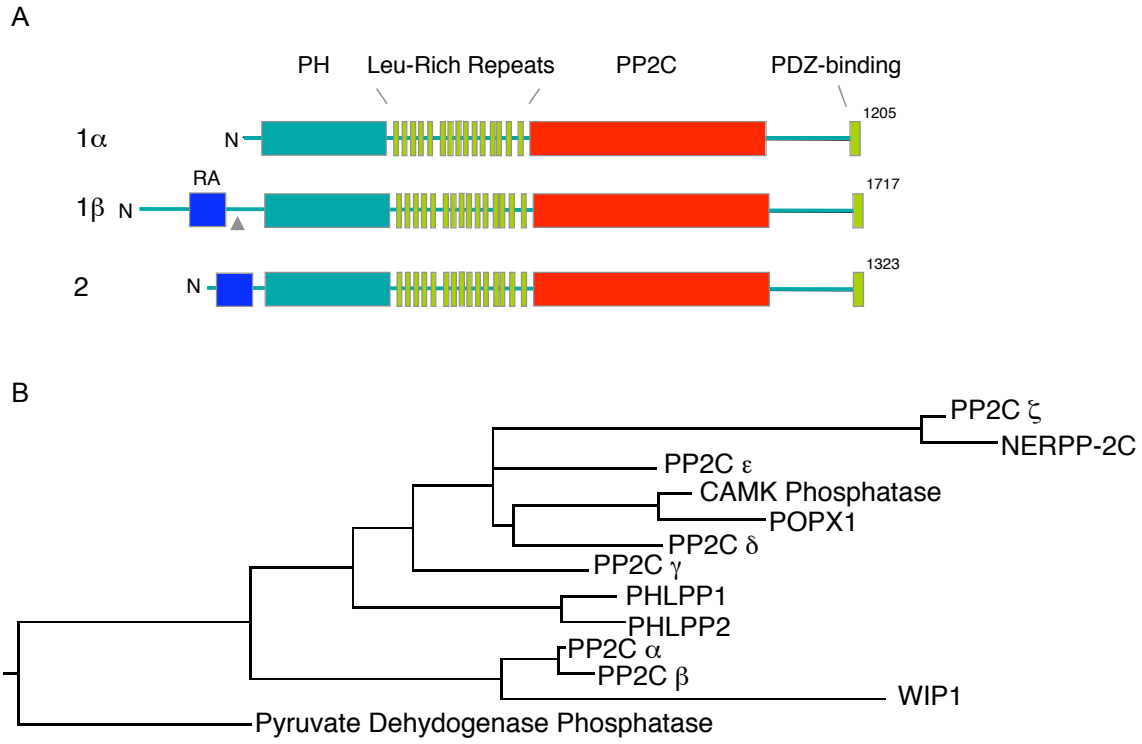

Figure 1: The PHLPP family of phosphatases**.** (A) Domain composition of PHLPP family members showing the Ras association domain (RA), PH domain, Leucine rich repeat (LRR) region, PP2C domain, and PDZ binding motif. (B) Phylogenetic tree of PP2C domains showing PHLPP is a discrete subfamily. Sequences were aligned using ClustalW (Gonnet matrix).

Table 1: PP2C domain alignment. Alignment of invariant aspartic acid residues in motifs 1,2,8, and 11, which are crucial for binding of metal ions. Alignment was performed using ClustalW identity matrix with only PP2C domains of phosphatases.

PP2C Phosphatase	1	2	8	11
PP2Cα (PPM1A)	RVEMED	FFAVYDGHAG	DGIWD	DNMSVILICF
PP2Cβ (PPM1B)	RVEMED	FFAVYDGHAG	DGIWD	DNMSVVLVCF
PP2Cγ (FIN13, PPM1G)	RVSMED	MFSVYDGHGG	DGIWN	DNMTCIIICF
PP2Cδ (ILKAP)	REEMQD	YFAVFDGHGG	DGLFK	DNVTVMVVRI
PP2Cε (PPM1L)	RDHMED	IFGIFDGHGG	DGLWD	DNITVMVVKF
PP2Cζ (PPM1J)	KSRHNED	YWGLFDGHAG	DGLWD	DDISVFVIPL
WIP1 (PPM1D)	RKYMED	FFAVCDGHGG	DGLWN	DNTSAIVICI
CaMKP (PPM1F)	RRKMED	YFAVFDGHGG	DGFFD	DNITVMVVFL
NERPP-2C (PPM1H)	KSTHNED	YWSLFDGHAG	DGLWD	DDISVYVIPL
POPX1 (PPM1E)	RRKMED	YFAVFDGHGG	DGFYD	DNITVIVVFL
PHLPP1 PP2C (SCOP)	QPSTGD	LYGVFDGDRN	DALAA	DSISAVVVQL
PHLPP2 PP2C (PHLPPL)	PLPTTD	VYGMFDGDRN	DPLAA	DNVGAMVVYL

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Chapter 2

PHLPP and a New Isoform, PHLPP2, Differentially Attenuate the Amplitude of Akt Signaling by Regulating Distinct Akt Isoforms

Summary

Ser/Thr kinase Akt/protein kinase B is a pivotal regulator of cell growth, proliferation and survival. We have recently discovered a novel phosphatase PHLPP, for PH domain Leucine-rich repeat Protein Phosphatase, which terminates Akt signaling by directly dephosphorylating and inactivating Akt. Here we describe a second family member, PHLPP2, which also inactivates Akt, inhibits cell cycle progression and promotes apoptosis. These phosphatases control the amplitude of Akt signaling: depletion of either isoform increases the magnitude of agonist-evoked Akt phosphorylation by almost two orders of magnitude. Although PHLPP1 and PHLPP2 both dephosphorylate the same residue (hydrophobic phosphorylation motif) on Akt, they differentially terminate Akt signaling by regulating distinct Akt isoforms. Knock-down studies reveal that PHLPP1 specifically modulates the phosphorylation of HDM2 and GSK-3α through Akt 2, whereas PHLPP2 specifically modulates the phosphorylation of p27 through Akt 3. In summary, our data unveil a new mechanism to selectively terminate Akt signaling pathways through the differential inactivation of specific Akt isoforms by specific PHLPP isoforms.

Introduction

Akt exquisitely controls the balance between cell survival and apoptosis, as well as proliferation and cellular quiescence. Signaling molecules that tip the balance towards survival, growth, and proliferation typically bind receptors that activate the lipid kinase, phosphatidylinositol 3-kinase (PI3K), resulting in production of 3'-phosphoinositide lipid second messengers, most notably phosphatidylinositol-3,4,5-trisphosphate (PtdIns P3) (1-3). This lipid product recruits Akt to the plasma membrane, where Akt is activated by two sequential phosphorylations: first by PDK-1 on the activation loop (Thr 308 in Akt1) followed by phosphorylation on the hydrophobic motif (Ser 473 in Akt1) (4). Phosphorylation of this second site has been proposed to be catalyzed by autophosphorylation (5), as it is for protein kinase C (6). However, it has been debated whether a unique kinase, tentatively referred to as "PDK-2", phosphorylates this site (7) and several candidates have been proposed (8-12), including most recently the TORC-2 protein complex (SIN1-rictor-mTOR) (13, 14).

The bis-phosphorylated species of Akt is locked in an active conformation that allows phosphorylation of substrates to promote cell growth, proliferation, and cell survival (15-18). Cell cycle effects are controlled through mechanisms that modulate the localization of cell cycle regulators such as p27 and the E3 ubiquitin ligase, HDM2 (human homologue to murine double minute 2). Akt directly phosphorylates p27, resulting in cytosolic sequestration of p27, promoting progression through the cell cycle (16, 17). Phosphorylation of HDM2 by Akt causes the ligase to translocate from the cytosol to the nucleus where it binds and inhibits the function of p53 by promoting

its translocation to the cytosol and subsequent ubiquitin-mediated degradation (19). Recent studies have identified TSC2 (tuberin) as a novel substrate for Akt. TSC2 is a tumor suppressor that forms a heterodimeric complex with TSC1 (hamartin) and is a GTPase activating protein (20) for the small G protein Rheb. When GTP is bound to Rheb it activates the TORC-1 complex (mTOR-raptor). Multi-site phosphorylation of TSC2 by Akt inhibits the GAP activity of TSC2 towards Rheb and thereby activates the TORC-1 complex resulting in increased activity of p70S6K and increased cellular growth. (21, 22). Akt mediates cell survival through phosphorylation of substrates such as GSK-3β (23-25) and members of the Forkhead Box O family of transcription factors (15, 26). The complexity of Akt signaling is underscored by reports that demonstrate distinct functions for each Akt isoform. Specifically, studies with knock-out mice have implicated Akt2 in glucose homeostasis and Akt1 in growth regulation (27, 28). Furthermore, recent reports reveal that Akt1 activation suppresses cell migration and invasion whereas Akt2 promotes invasion, particularly relevant in metastasis (29, 30). Thus, propagation of signaling by Akt is complicated and with multiple end points.

Akt signaling is terminated by a two-step mechanism: removal of the activating lipid second messenger and subsequent dephosphorylation of activated Akt. The lipid phosphatase PTEN directly dephosphorylates the 3' position of the inositol ring of PtdIns P3, ablating both recruitment of Akt to the plasma membrane and its subsequent activation (31). Direct dephosphorylation of Akt is mediated by phosphatases such as PP2A-type phosphatases (32, 33) and a novel phosphatase our lab recently discovered entitled PHLPP (34). PHLPP directly dephosphorylates the hydrophobic motif of Akt (Ser 473 on Akt1) resulting in inhibition of kinase activity and promotion of apoptosis (34). Given its role in terminating Akt signaling, PHLPP

is poised to be a potential tumor suppressor. Failure to terminate signaling via the PI3K/Akt pathway results in increased cell growth, proliferation, and inhibition of apoptosis. Loss of acute regulation of these cellular processes are hallmarks of tumorigenesis, and many proteins in this pathway, including PI3K and PTEN, are somatically mutated in cancer (18, 35).

Here we identify and characterize a second isoform of the PHLPP family, PHLPP2, and show that the PHLPP isoforms differentially terminate Akt signaling. Knock-down studies reveal that termination of defined Akt signaling pathways is mediated through the specific interactions of PHLPP1 and PHLPP2 with Akt1, 2, and 3. For example, PHLPP1 is in a complex with Akt2 that controls HDM2 and GSK-3α phosphorylation, and PHLPP2 is in a complex with Akt3 that controls p27 phosphorylation. These data suggest that signaling complexes of specific PHLPP isoforms, Akt isoforms, and substrates drives specificity in propagating and terminating signaling in the PI3K/Akt pathway.

Results

A search of the NCBI database for novel isoforms of the phosphatase PHLPP revealed a gene predicted to encode a 1323-residue protein that we name PHLPP2. The gene is located at chromosome 16q22.3 and is comprised of 18 exons. This gene is the only other gene predicted to encode a protein with the same domain composition as the originally described PHLPP: a PH domain, leucine-rich repeats, a PP2C phosphatase domain, and a PDZ-binding motif (Figure 1A). This new isoform shares 50% overall amino acid identity with the original PHLPP, which we hereafter refer to

as PHLPP1. Identity in the PH domains and PP2C domains of PHLPP1 and PHLPP2 are 63% and 58%, respectively. Key residues identified for phosphate and metal binding in PP2Ca are conserved in both PHLPP isoforms (asterisks in PP2C domain (yellow), Figure 1A) (36). It is also noteworthy that, similar to PHLPP1, only the second Arg of the signature motif RXRXF of phosphoinositide-binding PH domain is present in PHLPP2 (asterisk in PH domain (cyan), Figure 1A) (37). The most striking differences are an amino-terminal extension of approximately 14 kDa present in PHLPP2 and divergence in C-terminal sequences preceding the PDZ binding motifs.

Human PHLPP2 was cloned as described in Methods and HA-tagged PHLPP2 was expressed in H157 cells (Figure 1B, lane 6). Transiently-expressed HA-tagged PHLPP2 migrated with a molecular mass of approximately 150 kDa (Figure 1B lane 6), consistent with the additional 129 amino acids compared to HA-PHLPP1, which migrates with an apparent molecular mass of 140 kDa (Figure 1B, lane 7). To probe for expression of endogenous PHLPP1 and PHLPP2, we used isoform-specific antibodies (Figure 1B, lanes 1-5). PHLPP2-specific antibodies detected a band co-migrating with expressed PHLPP2 in 293T and H157 cells (Figure 1B, lanes 1 and 2) that was absent following knock-down of PHLPP2 (lane 5) but not PHLPP1 (lane 4) by isoform-specific siRNA. PHLPP1 was originally described as a 140 kDa protein, however the PHLPP1-specific antibody detected a major band with an apparent molecular mass of 190 kDa (Figure 1B, PHLPP1 panel, long exposure, double asterisk) and a minor band at 140 kDa (visible in long exposure, PHLPP1 panel, asterisk) consistent with the originally described PHLPP protein. Both bands were effectively depleted in cells treated with siRNA for PHLPP1, but not PHLPP2, suggesting the upper band is a splice variant of PHLPP1. In support of this, the most

recent update of gene annotation available through the NCBI database predicts a longer PHLPP1 gene (accession number O60346) containing extra 5' sequence upstream of the original PHLPP1 start codon (34) and thus encoding a larger protein (1717 residues). Consistent with two splice variants of PHLPP1, Northern blot analysis revealed expression of two transcripts of approximately 5 and 7 kb (data not shown). Thus, the PHLPP family comprises two gene products, PHLPP1 and PHLPP2, with PHLPP1 having 2 splice variants which we name PHLPP1α (the originally described PHLPP (34)) and PHLPP1β. Immunohistochemistry revealed that PHLPP1 and PHLPP2 are distributed throughout the cell in the Hs578Bst normal breast cell line (Figure 1C) and H157 cell line (data not shown) with fractionation studies supporting cytosolic, membrane, and nuclear distribution for both isoforms (data not shown).

We first tested whether the PP2C domain of PHLPP2 was catalytically competent. Figure 1D shows that the PHLPP2 PP2C domain catalyzed the dephosphorylation of the synthetic phosphatase substrate *para*-nitrophenylphosphate (*p*NPP) in a concentration- and time-dependent manner. Kinetic analysis revealed a K_m of 4.13 ± 0.05 nM and k_{cat} of 0.015 ± 0.001 s^{-1} (Figure 1E). This K_m is similar to that reported for PP2Cα towards *p*NPP, but the catalytic rate towards this synthetic substrate is approximately 100-fold lower than that of PP2Cα. We next asked whether the PP2C domain of PHLPP2, like that of PHLPP1, dephosphorylates Akt. Incubation of purified bacterially-expressed PHLPP2 PP2C domain with pure, phosphorylated Akt resulted in dephosphorylation of Ser 473 and Thr 308 as assessed with phospho-specific antibodies (Figure 1F; 82 ± 6% phospho-Ser 473 (P473) and 70 ± 2% of phospho-Thr 308 (P308) dephosphorylated in 5 minutes under the conditions

of the assay). Thus, the isolated PP2C domain of PHLPP2 encodes a functional phosphatase domain capable of dephosphorylating synthetic substrates as well as Akt *in vitro*.

We next addressed the phosphatase activity of full-length PHLPP2. In contrast to the isolated PP2C domain, immunoprecipitated full-length PHLPP2 specifically dephosphorylated Ser 473 and not Thr 308: incubation of immunoprecipitated HA-PHLPP2 with pure phosphorylated Akt resulted in 60 $\pm$ 4% dephosphorylation of Ser 473 under the conditions of the assay with no significant effect on Thr 308 phosphorylation (Figure 1G). These data reveal that inhibitory constraints imposed by the regulatory regions of PHLPP2 constrain its phosphatase activity so that it is specific for the hydrophobic motif of Akt.

PHLPP2 was also an effective Ser 473 phosphatase in cells: overexpression of HA-PHLPP2 in 293T (Figure 2A) and H157 cells (Figure 2B) resulted in a 76 ± 3% and 72 $\pm$ 8% reduction in phosphorylation at Ser 473, respectively, with minimal effects on the phosphorylation of Thr 308. Given transfection efficiencies of 70 – 90% for 293T cells, these data are consistent with the overexpressed PHLPP2 catalyzing the quantitative dephosphorylation of Akt in transfected cells, resulting in significantly decreased activity. Thus, PHLPP2 directly and selectively dephosphorylates the hydrophobic motif of Akt. (Note we chose to use the H157 cells (a non-small cell lung cancer cell line, NSCLC) because under conditions of serum deprivation, Akt inhibition has been shown to induce apoptosis (33, 38), thus providing a useful cell system to examine the effects of PHLPP2 on Akt-mediated apoptosis.)

To test whether endogenous PHLPP2 regulates the phosphorylation of Akt in cells, we knocked down endogenous PHLPP2 by siRNA. We generated three unique siRNAs and all resulted in greater than a 3.5 fold reduction in PHLPP2 protein in the

293T cells (Figure 2C, lanes 2 - 4); smartpool siRNA (combining all three siRNAs) was used for all subsequent experiments. Knock-down of PHLPP2 protein resulted in a 2-fold increase in Akt phosphorylation at Ser 473, but did not significantly alter phosphorylation at Thr 308 in 293T cells (Figure 2C lanes 2-4). Knock-down of PHLPP2 resulted in a 4-fold increase in Ser 473 phosphorylation and no change in Thr 308 phosphorylation in H157 cells (Figure 2C, lane 6). Similar results were obtained following knock-down of PHLPP2 in SKBR-3 and MCF-7 cells (Supplementary Figure 1).

Maximal Akt activity requires phosphorylation on both Ser 473 and Thr 308, leading us to address the effect of dephosphorylation by PHLPP2 on cellular Akt activity. Akt immunoprecipitated from 293T cells overexpressing PHLPP2 had markedly reduced levels of Ser 473 phosphorylation compared to Akt from vector-transfected cells (Figure 2D; 70% reduction in Ser 473 phosphorylation); this reduced phosphorylation correlated with reduced activity towards phosphorylation of a GSK-3 fusion protein substrate in an *in vitro* kinase assay (Figure 2D; 80% reduction in substrate phosphorylation). Thus, the selective dephosphorylation of Ser 473 on Akt by PHLPP2 results in a dramatic decrease in kinase activity.

To determine if Akt and PHLPP2 associate in cells, we immunoprecipitated HA-PHLPP2 from 293T cells and probed for association with endogenous Akt. Figure 2E shows that endogenous Akt was present in immune complexes of HA-tagged PHLPP2, revealing that the two proteins associate in cells.

We next addressed the specificity of PHLPP2 for the hydrophobic phosphorylation motif (Ser 473) of Akt relative to that of the other AGC family kinase members: 70 kDa ribosomal S6 kinase (p70S6K), 90 kDa ribosomal S6 kinase (p90RSK), and protein kinase C (PKC). Expression of PHLPP2 caused a marked decrease in the phosphorylation of Akt on Ser 473, but, in the same cells, had no

significant effect on the phosphorylation of the hydrophobic motif of PKC, p90RSK, or p70S6K (Figure 2F). These data are consistent with PHLPP2 specifically dephosphorylating the hydrophobic motif of Akt under the conditions of our experiments. It was previously reported that a protein corresponding to PHLPP1 negatively regulates the MAPK signaling pathway (39). To determine whether PHLPP2 also negatively regulates the MAPK signaling pathway, we examined activation of this pathway in untreated cells expressing empty vector or HA-PHLPP2. PHLPP2 overexpression did not alter the phosphorylation of MEK 1/2 or ERK 1/2 under the conditions of the experiment (Figure 2F), suggesting PHLPP2 does not regulate this pathway.

The foregoing results reveal that PHLPP isoforms suppress the phosphorylation of Akt under basal conditions by selectively dephosphorylating Ser 473. We next asked whether the PHLPP isoforms control the amplitude or duration of agonist-stimulated Akt phosphorylation. Treatment of Hs578Bst, a normal breast cell line, with EGF resulted in a rapid and transient rise in the phosphorylation state of Akt on Ser 473 and Thr 308: Figure 3A shows that the phosphorylation at both sites increased significantly following 15 min of EGF treatment (lane 2) but returned to baseline following 30 min treatment (lane 3). Depletion of both PHLPP1[1] and PHLPP2 resulted in a remarkable 30-fold increase in the EGF-stimulated phosphorylation of Akt on Ser 473 and, unexpectedly, Thr 308 (Figure 3A, lane 8). This increase was sustained longer relative to control cells, with decay to PHLPP depleted base line levels requiring at least 24 hours for Ser 473 (lane 12). Curiously, PHLPP isoforms selectively control the phosphorylation state of Ser 473 under basal conditions (10% FBS DMEM), but control the phosphorylation state of Ser 473 and

[1] Because siRNA depletes both PHLPP1α and PHLPP1β, we use PHLPP1 to denote both PHLPP1α and PHLPP1β.

Thr 308 following acute agonist-stimulation. These data establish that PHLPP isoforms play a major role in controlling the amplitude of agonist-dependent phosphorylation of Akt. Similar, but less robust results were observed in the H157 cell line (data not shown).

We next compared the effects of depletion of PHLPP1 or PHLPP2 individually on the agonist-dependent phosphorylation of Akt. Consistent with the results in Figure 2C for 293T and H157 cells, depletion of either PHLPP1 (lane 2), PHLPP2 (lane 3) or both (lane 4) in Hs578Bst cells caused an increase in the basal phosphorylation state of Ser473, but not Thr308 (Figure 3B). Stimulation with EGF for 15 min caused an increase in the phosphorylation of Ser473 and Thr308 (lane 5) that was highly dependent on PHLPP1 and PHLPP2: depletion of either isoform caused a striking increase in the phosphorylation of Ser473 and Thr308. Knock-down of both isoforms resulted in a comparable increase to that observed following single knock-down. Thus, both PHLPP1 and PHLPP2 set the amplitude and duration of the Akt signal.

The finding that dephosphorylation by PHLPP2 inactivates Akt led us to hypothesize that the cellular processes controlled by Akt are regulated by PHLPP2. To address this possibility, we examined the effects of expressing PHLPP2 on Akt-mediated apoptosis. Expression of PHLPP2 resulted in an increase in apoptosis in H157 cells under conditions of serum deprivation: the relative sub-2N DNA increased an order of magnitude from 3.1 ± 0.1% in vector-transfected cells to 28 ± 5% in PHLPP2-transfected cells (Figure 4A). To examine if PHLPP2 regulated Akt-mediated apoptosis in other cancer cell lines, we expressed HA-PHLPP2 in the Bt-474 and MDA-MB-231 breast cancer cell lines: expression of PHLPP2 resulted in an approximately 80% and 70% decrease in phosphorylation at Ser 473, respectively (Figure 4B). Comparable results were observed in the ZR-75-1 and MCF-7 breast

cancer cell lines (data not shown). Furthermore, expression of PHLPP2 in breast cancer cells resulted in an increase in apoptosis (from 2.0 $\pm$ 0.2 to 15.8 $\pm$ 0.6 relative units in Bt-474 cells and from 2.9 $\pm$ 0.9 to 12 $\pm$ 4 relative units in MDA-MB-231 cells), as assessed by quantifying sub-2N-DNA content (Figure 4B). These data reveal that, as noted previously for PHLPP1, PHLPP2 also promotes apoptosis.

To more rigorously explore the role of the PHLPP isoforms in mediating apoptosis, we examined the effect of depletion of PHLPP1, PHLPP2, or both isoforms on apoptosis triggered by the DNA-damaging agent etoposide. Treatment of H157 cells with etoposide resulted in a 5-fold increase in apoptosis (Figure 4C). Depletion of PHLPP1, PHLPP2, or both isoforms caused a 2-fold reduction in epotoside-mediated apoptosis. These results reveal that PHLPP isoforms promote apoptosis both under basal conditions and following exposure to cytotoxic agents.

We next tested whether the increased apoptosis observed in cells overexpressing PHLPP2 resulted from PHLPP2-mediated dephosphorylation of Ser 473 on Akt. To this end, we co-expressed PHLPP2 with a phosphomimetic, and thus constitutively-active, Akt construct (S473D) in MDA-MB-231 cells. Importantly, we found that the PHLPP2-resistant Akt rescued two-thirds of the apoptosis induced by PHLPP2 (Figure 4D). Thus, PHLPP2 negatively regulates Akt resulting in the induction of apoptosis, and this effect can be rescued by a phosphomimetic Akt construct resistant to dephosphorylation by PHLPP2.

Akt has been reported to regulate both proliferation and cell cycle entry leading us to ask whether PHLPP2 affects cell cycle progression. Expression of PHLPP2 resulted in an approximately 3-fold and 2-fold increase in the G1/S ratio (as assessed by flow cytometry) in both 293T and H157 cells, respectively (Figure 4E), suggesting cells were entering the cell cycle at a decreased rate. Consistent with this

result, knock-down of endogenous PHLPP2 in H157 cells caused a 2-fold decrease in the G1/S ratio indicating that the cells in which PHLPP2 was depleted were proliferating at an increased rate (Figure 4F). Interestingly, despite knock-down of either PHLPP1 or PHLPP2 causing a comparable increase in Ser 473 phosphorylation (see Figure 4G and 5A-C), PHLPP1 depletion resulted in a smaller decrease (approximately 25%) in the G1/S ratio compared to the approximately 50% decrease resulting from PHLPP2 depletion. Consistent with decreased levels of PHLPP2 causing a selective increase in cell proliferation in the H157 cells, BrdU incorporation increased 1.7-fold in cells in which PHLPP2 was knocked down and only 1.3-fold in cells in which PHLPP1 was knocked down (Figure 4G). The combined knock-down of both PHLPP1 and PHLPP2 did not cause BrdU incorporation to differ significantly from the increase resulting from PHLPP2 knock-down alone. Importantly, similar increases in BrdU incorporation were observed in the normal breast cell line, Hs578Bst (Figure 4G). Western blot analysis of the cells used for the BrdU analysis verified that PHLPP1 and PHLPP2 had been selectively knocked-down and revealed that Ser473 phosphorylation increased for both knock-downs (Figure 4G). These data reveal that both PHLPP1 and PHLPP2 control cell proliferation by regulating the activation state of Akt, with PHLPP2 having a more pronounced effect.

To address the mechanism driving the more pronounced effects on the cell cycle by PHLPP2 compared with PHLPP1, we examined the effect of knocking down each PHLPP isoform alone or in combination on the phosphorylation state of downstream substrates of Akt. Figure 5A shows that PHLPP1 (lane 2) and PHLPP2 (lane 3) were effectively and specifically knocked-down with isoform-specific siRNA treatment in H157 cells, resulting in a 5-fold increase in the phosphorylation of Ser 473 (lanes 2 and 3, P473 panel; data from 3 independent experiments quantified in Figure 5C). Knock-down of both isoforms did not further increase Ser 473

phosphorylation relative to selective knock-down of each isoform individually (lane 4, P473 panel). Knock-down of PHLPP1, but not PHLPP2, specifically increased the phosphorylation of two Akt substrates: HDM2 (P-HDM2 panel; phosphorylation of Ser166) and GSK-3α (P-GSK-3α; phosphorylation of Ser21). Knock-down of PHLPP2, but not PHLPP1, specifically increased the phosphorylation of p27 on Thr157 (P-p27 panel). Knock-down of either PHLPP1 or PHLPP2 increased the phosphorylation of GSK-3β (P-GSK-3β; phosphorylation of Ser9), and TSC2 (P-TSC2; phosphorylation on Ser 939 and Thr 1462) (Figure 5A and 5B, respectively). Interestingly, knock-down of either PHLPP1 or PHLPP2, or both isoforms, did not significantly alter the phosphorylation of FoxO1 on Ser256 (Figure 5A; P-FoxO1). However, knock-down of either isoforms caused an increase in the phosphorylation of Thr24 on FoxO1 (Figure 5B, P-FoxO1 Thr24 panel) that was more significant for the PHLPP2 knock-down compared to the PHLPP1 knock-down (Figure 5C). The results of 3 independent experiments are quantified in Figure 5C. These data reveal that although PHLPP1 and PHLPP2 both dephosphorylate the same residue on Akt, a subset of downstream targets of Akt are differentially modulated depending on which phosphatase is depleted.

We next tested the hypothesis that the PHLPP isoforms differentially regulate distinct Akt isoforms, providing a possible mechanism underlying differences in substrate regulation. In this regard, Akt isoforms have been reported to regulate unique downstream substrates (27, 28, 40). To address this, we depleted cells of PHLPP1, PHLPP2 or both isoforms, immunoprecipitated each Akt isoform, and examined the phosphorylation of Ser 473. Western blot analysis of Akt immunoprecipitates (Figure 6A) revealed that knock-down of PHLPP1 caused an increase in the phosphorylation of the hydrophobic motif of Akt2 (lane 6, P473 panel)

but not Akt1 (lane 2, P473 panel). Conversely, knock-down of PHLPP2 caused an increase in the phosphorylation of the hydrophobic motif of Akt1 (lane 3, P473 panel) but not Akt2 (lane 7, P473 panel). Knock-down of either PHLPP isoform increased the phosphorylation of the hydrophobic motif of Akt3 (lanes 10 and 11, P473 panel). Note that under these conditions activation loop phosphorylation was not affected by knock-down of individual PHLPP isoforms (P308 panel). These data reveal that PHLPP2 controls the phosphorylation of the hydrophobic motif on Akt1 and Akt 3 and PHLPP1 controls the phosphorylation of the hydrophobic motif on Akt2 and Akt3.

We also examined whether PHLPP isoforms selectively interacted with Akt isoforms in cells by immunoprecipitating endogenous Akt isoforms and probing for endogenous PHLPP isoforms. Supporting the results on effects of PHLPP isoforms on hydrophobic motif phosphorylation of Akt isoforms, PHLPP1 immunoprecipitated with Akt2 and Akt3 (Figure 6A, PHLPP1 panel; lanes 5/7 and 9/11), whereas PHLPP2 immunoprecipitated with Akt1 and Akt3 (Figure 6A, PHLPP2 panel; lanes 1/2 and 9/10). Note that this selectivity was lost in overexpression studies: overexpressed PHLPP1 and PHLPP2 bound all 3 Akt isoforms (Supplemental Figure 2). Thus, the data presented in Figure 6A establish that: 1] Akt1 binds to and is specifically dephosphorylated at the hydrophobic motif by PHLPP2, 2] Akt2 binds to and is specifically dephosphorylated at this motif by PHLPP1, and 3] both PHLPP isoforms bind and regulate the dephosphorylation of Akt3.

To test whether the isoform-specific effects of PHLPP knock-down on Akt substrates resulted from differential dephosphorylation of Akt isoforms, we depleted each Akt isoform by specific siRNA and examined the phosphorylation status of downstream substrates. The Western blot in Figure 6B shows that knock-down of Akt 2 (lane 3), but not Akt 1 (lane 2) or Akt 3 (lane 4) resulted in a decrease in

phosphorylation of HDM-2 (Ser 166) and GSK-3α (Ser 21), in agreement with the increases observed following knock-down of PHLPP1 (Figure 5A). Depletion of Akt3 (lane 4), but not Akt1 or Akt2, resulted in a dramatic decrease in p27 phosphorylation (Thr 157), in agreement with the robust increase in phosphorylation following knock-down of PHLPP2. Interestingly, although PHLPP1 also regulates Akt3, PHLPP1 knock-down did not effect p27 phosphorylation (Figure 5A). Perhaps unique spatial regulation within the cell controls specificity in the PHLPP2-Akt3-p27 pathway. Depletion of each Akt isoform resulted in a decrease in GSK-3β phosphorylation, consistent with PHLPP knock-down studies showing that both isoforms of the phosphatase increase the phosphorylation of this substrate (Figures 5A and 6B). The phosphorylation of Thr 24 on FoxO1 was decreased following knock-down of either of the 3 Akt isoforms whereas the phosphorylation of Ser 256 was unaffected. These results are consistent with the PHLPP knock-down results: phosphorylation of Thr 24, but not Ser 256, was increased following PHLPP knock-down (Figure 5C). Lastly, knock-down of Akt 1 and Akt2, but not Akt3, caused a decrease in the phosphorylation of both Ser 939 and Thr 1462 on TSC2, again in agreement with the effects of PHLPP knock-down. Note that depletion of Akt1 did not decrease total Ser 473 phosphorylation significantly (lane 2, Ser 473 panel), likely due to up-regulation of Akt2 (compare lanes 1 and 2; Akt2 panel). Data from 3 independent experiments are quantified in Figure 6C. In summary, results from the complementary approaches of knock-down of PHLPP isoforms or knock-down of Akt isoforms converge on a unifying model in which PHLPP1 regulates Akt2 and Akt3 whereas PHLPP2 regulates Akt1 and Akt3. Thus, PHLPP1 selectively controls signal termination via the HDM2, GSK-3α/β, TSC2 and FoxO1 pathways by inactivating Akt2 and Akt3, whereas PHLPP2 controls signal termination via the p27, GSK-3β, TSC2, and FoxO1

pathways by inactivating Akt 1 and Akt3.

As a test of the model that PHLPP isoforms differentially inactivate Akt isoforms thus differentially terminating signaling pathways, we asked whether the effects of a specific PHLPP isoform would be abolished if its partner, Akt, was also depleted. We chose to test the model with the PHLPP2-Akt3-p27 signaling complex because the specificity of this particular interaction [only PHLPP2 (not PHLPP1) and only Akt3 (not Akt1 or Akt2) controls p27 phosphorylation] under agonist-stimulated conditions in the Hs578Bst primary breast cell line. Figure 6D shows that knock-down of PHLPP2 caused an increase in the phosphorylation of p27 on Thr 157 (lane 3) relative to control (lane 1) or cells depleted in Akt3 (lane 2). This increase was abolished upon knock-down of both PHLPP2 and Akt3 (lane 4). These data reveal that the ability of PHLPP2 to modulate the phosphorylation of p27 depends on Akt3, supporting the model that specific PHLPP isoforms control the activity of specific Akt isozymes, in turn controlling the phosphorylation of specific downstream substrates.

An intriguing finding from the foregoing experiments was that simultaneous knock-down of both PHLPP isoforms did not result in an additive increase in Ser 473 phosphorylation or the phosphorylation of downstream substrates of Akt (see quantitation in Figure 5C: knock-down of either PHLPP1, PHLPP2, or both PHLPP isoforms caused a 5-fold increase in Ser473 phosphorylation and, for example, a 4-fold increase in GSK-3β phosphorylation). One possibility is that depletion of both PHLPP isoforms activates an inhibitory feedback signal. A candidate for such feedback regulation is p70S6K, whose activation has been established to result in decreased phosphorylation and activation of Akt (41-43). To test this possibility, we asked whether inhibition of p70S6K activation by rapamycin (TORC-1 inhibitor) unmasked any potential additivity in the knock-down of PHLPP1 and PHLPP2.

Figure 6E shows that, as presented in earlier experiments, knock-down of PHLPP1 (lane 2), PHLPP2 (lane 3), or both PHLPP isoforms (lane 4) resulted in a comparable increase in Ser473 phosphorylation. Knock-down of either PHLPP1 or PHLPP2 had no significant effect on the phosphorylation of Thr 308, however a modest decrease in Thr 308 phosphorylation was observed when both PHLPP isoforms were depleted (P308 blot, lane 4). Treatment of cells with rapamycin resulted in decreased overall Ser 473 phosphorylation, however depletion of either PHLPP1 or PHLPP2 increased Ser 473 phosphorylation in rapamycin-treated cells (lanes 6 and 7). In marked contrast to untreated cells, rapamycin treatment doubled the level of Ser 473 phosphorylation in cells in which both PHLPP isoforms had been knocked down (lane 8) compared to cells in which the PHLPP isoforms had been knocked down individually (lanes 6 and 7). Additionally, the phosphorylation of Thr 308 was no longer reduced in the double-knock-down cells compared to the single knock-down cells (compare lane 8 to lanes 6 and 7). Note that the degree of Ser 473 phosphorylation (but not Thr 308) was generally reduced in rapamycin-treated cells (compare lanes 1-4 to lanes 5-8). This result could be explained by newly synthesized mTOR binding rapamycin, thus decreasing the levels of the TORC-2 complex, which would result in a decrease in Ser 473 phosphorylation (44). Consistent with activation of a p70S6K feedback loop when both PHLPP isoforms are depleted, knock-down of PHLPP1 or PHLPP2 alone did not dramatically increase p70S6K phosphorylation, but knock-down of both isoforms resulted in a robust increase in phosphorylation of this kinase (Figure 6E). In summary, these data reveal that depletion of both PHLPP

isoforms activates a negative feedback loop mediated by p70S6K that counteracts the direct effects of PHLPP depletion on Ser 473 phosphorylation.

Discussion

Here we identify a second isoform of the protein phosphatase PHLPP, which we name PHLPP2. We show that both PHLPP1 and PHLPP2 selectively dephosphorylate the same site on Akt, the hydrophobic phosphorylation motif, yet the two phosphatases control different downstream substrates of Akt. We identify the mechanism for the differential signal termination as deriving from specificity in the binding and regulation of specific PHLPP isoforms with specific Akt isoforms. Thus, for example, the phosphorylation state of p27 phosphorylation is controlled by a PHLPP2:Akt3 complex, whereas the phosphorylation state of HDM2 is controlled by a PHLPP1:Akt2 complex. This work unveils a novel mechanism to selectively control the amplitude of signaling pathways mediated by Akt via interaction of specific Akt isoforms with specific PHLPP isoforms and, presumably, specific substrates.

PHLPP2 dephosphorylates and inactivates Akt. Biochemical and cellular analyses reveal that PHLPP2, like PHLPP1, selectively dephosphorylates the hydrophobic motif of Akt, resulting in decreased kinase activity, increased apoptosis, and inhibition of cell cycle progression. Although the isolated PP2C domain is capable of dephosphorylating Thr 308 and Ser 473, the full-length protein has specificity for Ser 473. These data reveal that the regulatory domains of PHLPP constrain substrate phosphorylation, resulting in the full-length protein discriminating

between phosphorylation sites within the kinase core of Akt. The primary mechanism for the cellular effects of PHLPP2 presented are consistent with direct dephosphorylation of Ser 473 of Akt because the phosphomimetic, Akt S473D, is able to rescue the effects of PHLPP2 overexpression (see Figure 4D). Although a number of AGC kinases share the hydrophobic phosphorylation motif, under the conditions of our experiments (high serum growth conditions), we show that overexpressed PHLPP2 does not dephosphorylate the hydrophobic motif of PKC, p70S6K, or p90RSK. Nor does it regulate the MEK/ERK pathway. Thus, PHLPP2 directly and specifically regulates Akt under the conditions described.

PHLPP isoforms control the amplitude of agonist-dependent signaling by Akt. Depletion of either PHLPP1 or PHLPP2 causes a dramatic increase in the agonist-stimulated phosphorylation of Akt. In the normal breast cell line, Hs578Bst, depletion of either PHLPP isoform results in a 30-fold increase in the phosphorylation of Ser 473, relative to control cells, following EGF stimulation. These data reveal that PHLPP isoforms provide the brakes to agonist-mediated signaling: they directly set the amplitude of the signal by controlling the phosphorylation state of Ser 473. Interestingly, although the dephosphorylation of Akt following agonist stimulation was slowed in cells lacking PHLPP, the phosphorylation of Akt returned to PHLPP-depleted basal levels. These data suggest that an additional phosphatase is involved in controlling the duration of agonist-evoked activation of Akt in absence of the PHLPP phosphatases. One possibility is that a phosphatase directed at the PDK-1 site (activation loop, Thr 308) drives this de-activation with dephosphorylation at this site

destabilizing the phosphate at the hydrophobic motif. A likely candidate is a PP2A-type phosphatase, which has been shown to regulate Akt phosphorylation (Andjelkovic et al., 1996; Yoshizaki et al., 2004). Note that the phosphorylation of the PDK-1 site has marked sensitivity to okadaic acid (34); the hydrophobic site (Ser 473) is only modestly affected by okadaic acid, consistent with a PP2C family member controlling this site. Live cell imaging studies of Akt activity have previously established that phosphatases are powerful 'breaks' to Akt signaling (45). Here we show that PHLPP isoforms exert enormous suppression on the acute agonist-mediated phosphorylation of Akt, thus setting the amplitude of the signal.

Curiously, the phosphorylation state of both Thr 308 and Ser 473 were coordinately elevated following acute agonist stimulation of cells lacking PHLPP1 or PHLPP2. In contrast, only the phosphorylation state of Ser 473 was affected in cells lacking PHLPP isoforms under basal conditions. One possibility is that the phosphorylation state of the hydrophobic motif regulates the stability of the phosphate on the activation loop. This is indeed the case with Akt's close cousin, protein kinase C: negative charge at the hydrophobic site renders the phosphate on the activation loop relatively resistant to dephosphorylation (46, 47). Evidence suggests this could be the case for Akt: mutation of Ser 473 to Asp results in a construct of Akt that is more heavily phosphorylated on Thr 308 compared to wild-type Akt (48). Thus, it may be that the enormous increase in Ser 473 phosphorylation resulting from depletion of PHLPP stabilizes the activation loop site, rendering it less sensitive to cellular phosphatases. This stabilization may not be apparent under basal conditions

because the elevation in Ser 473 phosphorylation under these conditions is one order of magnitude lower than the agonist-stimulated elevation in Ser 473 phosphorylation. Another possibility is that increased phosphorylation at Ser 473 confers a more favorable conformation for PDK-1 phosphorylation; in support of this hypothesis, mutation of Ser 473 to Asp increases PDK-1 phosphorylation at Thr 308 in *in vitro* kinase assays (48). This interplay between the two phosphorylation sites is consistent with X-ray structure of Akt, which suggests that phosphorylation of the hydrophobic motif orders the activation loop (49, 50).

PHLPP isoforms differentially regulate signaling by Akt isoforms. Akt regulates cellular proliferation and apoptosis through multiple mechanisms initiated by its phosphorylation of precise downstream substrates. Here we define signaling networks regulated by unique Akt isoforms whose amplitude is controlled by specific PHLPP isoforms (Figure 7). Knock-down studies reveal that PHLPP2 is the dominant phosphatase in controlling the cell cycle. Furthermore, it specifically opposes the action of Akt3 on the phosphorylation state of p27: knock-down of Akt3 (but not Akt1 or 2) decreased p27 phosphorylation whereas knock-down of PHLPP2 (but not PHLPP1) increased p27 phosphorylation. Specific signaling scaffolds are implicated by our finding that PHLPP1 binds and dephosphorylates Akt3, yet p27 phosphorylation is not sensitive to PHLPP1 depletion. Thus, a PHLPP2-Akt3-p27 pathway is suggested by our data. PHLPP1, on the other hand, regulates the phosphorylation of HDM2 and GSK3-α, and our data suggest that the mechanism is by dephosphorylation of Akt2. Specific regulation of GSK3-α by Akt 2 has been

previously reported (40). We also identify a group of substrates whose phosphorylation is controlled by all three Akt isoforms and both PHLPP isoforms. For example, the phosphorylation state of GSK-3β and FoxO1 increases following knock-down of either PHLPP isoform and decreases following knock-down of any of the Akt isoforms. The overlapping regulation of these proteins by both PHLPP isoforms is consistent with the apoptotic effects induced by both phosphatases. Curiously, our data reveal that PHLPP and Akt selectively regulate specific residues within the same protein substrate: under conditions where the phosphorylation state of FoxO1 at Thr 24 was impacted by PHLPP knock-down, no effects on the phosphorylation of Ser 256 were observed. These data are consistent with a recent report showing that the phosphorylation of some Akt substrates is independent of the phosphorylation state of Ser 473 (14). In particular, the phosphorylation state of FoxO1 on Ser256 was reported to be the same in normal cells and in SIN1 knock-down cells (where Ser 473 phosphorylation is abolished) (14). The possibility that the species of Akt phosphorylated at Thr 308, but not Ser 473, has activity towards some substrates adds a new level of fine-tuning to signal control by the PHLPP isoforms. Lastly, we found some substrates that displayed intermediate specificity. The phosphorylation state of TSC2 was affected by a PHLPP2/Akt1 and by PHLPP1/Akt2 but was relatively insensitive to Akt3. These data underscore the role of isoform specificity in driving downstream signaling of the PI3K/Akt pathway.

Whether specific PHLPP isoforms are scaffolded with specific Akt isoforms and their substrates via protein scaffolds, or whether specific PHLPP isoforms bind

specific Akt isoforms via direct interactions, remains to be determined. The possibility that scaffold proteins control the interaction of PHLPP with Akt is supported by the finding that deletion of the PDZ-binding motif of PHLPP1 abolishes the ability of this isoform to dephosphorylate Akt in cells (34). Whether the spatial segregation of PHLPP1 and PHLPP2 signaling pathways is driven by the different PDZ-binding motifs in the two isoforms remains to be determined. Alternatively, specific PHLPP isoforms could bind specific Akt isoforms directly, possibly mediated by the linker regions in Akt, which are unique to each isoform (51).

Feedback regulation of Akt signaling activated by depletion of both PHLPP isoforms. Our data also reveal that the simultaneous knock-down of both PHLPP1 and PHLPP2 activates the well-characterized feedback regulation of Akt mediated by p70S6K (41-43). Thus, although PHLPP1 and PHLPP2 control different Akt isozymes, depletion of both isoforms does not enhance Akt phosphorylation relative to single knock-down because Akt phosphorylation is more robustly inhibited by activation of the p70S6K mediated feedback loop. This feedback inhibition is suppressed by rapamycin, allowing additivity in the effects of knock-down of PHLPP1 and PHLPP2. These results underscore the compensatory mechanisms that constrain perturbations to signaling pathways. In the case of PHLPP, lack of both isoforms increases activation of a negative feedback loop that dampens the phosphorylation of Akt in a manner that counteracts the profound effects of losing both phosphatases.

Summary. Our data reveal that PHLPP1 and PHLPP2 selectively dephosphorylate specific Akt isoforms, thus differentially controlling the amplitude of

Akt signaling. Taken together with results from Akt knock-out mice, it is intriguing to suggest that PHLPP1 plays a role in glucose homeostasis (where Akt2 is critical) whereas PHLPP2 plays a role in cell survival (where Akt1 is critical) (52). Our data also underscore the role of spatial segregation (i.e. via specific signaling complexes such as PHLPP1:Akt2 and PHLPP2:Akt1) in driving specificity in both signal propagation and signal termination in the PI3K/Akt pathway.

Experimental Procedures

Materials and Methods. PHLPP2-specific siRNA was purchased from Dharmacon and targeted the following sequences: 5'-CCTAAGTGGCAACAAGCTT-3' (si-1); CCATTCAAGATGAGTTGCT (si-2); and GGACAGCCTGAACCTCATTG (si-3) in PHLPP2. All three siRNAs resulted in greater than a 3.5 fold reduction in PHLPP2 protein in 293T cells (data not shown); smartpool siRNA (combining all three siRNAs) was used for all experiments except Figure 1C, which used si-1.) Smartpool siRNA against PHLPP1 and PHLPP2 was also purchased from Dharmacon. Akt isoform-specific siRNA was purchased from Santa Cruz.

Cloning and expression. Full length PHLPP2 cDNA was cloned by combining the bf979574 cDNA (I.M.A.G.E. Consortium (sequencing revealed the C-terminus of construct was same as BC035267 cDNA)) and AB023148 cDNA (Kazusa cDNA collection). Five nucleotides were not present in bf979574 cDNA based on the predicted PubMed sequence, NM_015020, and these nucleotides were added using the

Quik-Change Site-Directed Mutagenesis Kit (Stratagene). The nucleotide sequence of the resulting two constructs (bf979574 and AB023148) corresponded to the predicted sequence NM_015020, resulting in the amino acid sequence in Figure 1A. Because of a discrepancy in the amino acid at position 542 in the predicted sequence of NM_015020 (Val) with that of Sequence ID Q6ZVD8 (Leu), we sequenced 7 cell lines (H157, 293T, MDA-MB-231, ZR-75-1, MCF-7, SK-BR-3, and T47D) and confirmed that Leu is the correct residue. To express HA-tagged full length PHLPP2, sequence was amplified by PCR and subcloned into *Not I* and *Xba I* sites in the pcDNA3HA vector (34). A GST-tagged construct of the PP2C domain for bacterial expression was generated by amplifying the coding region of the PP2C domain (corresponding to residues 780-1030) by PCR and sub-cloning the sequence into *EcoR I* and *Xho I* sites of pGEX-KG vector (53).

Cell transfections and immunoblotting. ZR-75-1 and T47D cell lines were maintained in RPMI 1640 (Cellgro), and all other cell lines were maintained in DMEM (Cellgro); both media were supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained at 37ºC in 5% CO_2. Transient transfections and siRNA experiments were performed as previously described (34), except for ZR-75-1 cells, which were transfected using FuGENE6 reagent (Roche). Transfection efficiencies for 293T and H157 cell lines averaged between 70-90% for each experiment; efficiencies for breast cancer cell lines averaged between 50-85%. For immunoblotting, transfected cells were lysed in Buffer 1 (50 mM Na_2HPO_4, pH 7.5, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% SDS,

1 mM DTT, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, and 1 mM PMSF) and sonicated for 5 seconds. Lysates containing equal protein were analyzed on SDS-PAGE gels, and individual blots were probed using each antibody. Densitometric analysis was performed with the NIH Image analysis software (version 1.63).

Phosphatase assays and co-immunoprecipitations. GST-PP2C was expressed in BL21 bacterial cells; phosphatase assays using *p*NPP as substrate were performed using the purified GST-PP2C construct in buffer containing 0.05 M Tris, 0.05 M Bis-Tris, 0.1 M sodium acetate, pH 7.5, at 28 °C. Dephosphorylation of *p*NPP was measured by continuously monitoring the change in absorbance at 405 nM using a Thermo Electron Corp. Genesys 10 UV-visible spectrophotometer. Initial rates were determined using the molar extinction coefficient (_) of 12.8 $mM^{-1}.cm^{-1}$ for the product *para*-nitrophenol (*p*NP) at pH 7.5. To determine the kinetic parameters k_{cat} and k_{cat}/K_m, the initial velocities were measured at various substrate concentrations and the data were fit to the Michaelis-Menten equation by non-linear regression analysis. Phosphatase assays were also performed using the GST-PP2C construct conjugated to glutathione-sepharose and pure Akt1 as a substrate as previously described (34). The activity of full length HA-PHLPP2 was assessed by expressing and immunoprecipitating PHLPP2 from 293T or H157 cell lysates. Cells were lysed in Buffer 2 (20 mM HEPES, pH 7.4, 1% Triton X-100, 1 mM DTT, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, and 1 mM PMSF). Detergent soluble lysates were incubated overnight at 4° C with HA antibody and ultra-link protein A/G beads (Pierce). Beads were then washed three times with Buffer 1 and incubated in

phosphatase buffer with purified phosphorylated Akt as previously described (34). HA-PHLPP2 was immunoprecipitated from 293T cells as described above and the cells were lysed in Buffer 3 (50 mM Na_2HPO_4, pH 7.5, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM DTT, 200 μM benzamidine, 40μg ml^{-1} leupeptin, and 1 mM PMSF) and washed in Buffer 3 four times. Akt agarose was used to immunoprecipitate endogenous Akt (Upstate Biotechnologies). Akt isoforms were immunoprecipitated from H157 cells as described above using isoform-specific antibodies (Cell Signaling), washed four times in Buffer 3, and probed with pan or phospho-specific antibodies as well as PHLPP1 or PHLPP2 specific antibodies from Bethyl Laboratories.

In vitro kinase assay. Akt was immunoprecipitated from cell lysates using Akt agarose and kinase reactions were performed using an Akt kinase assay kit (Cell Signaling) as described previously (38).

Immunofluorescence Staining. Hs578Bst and H157 cells were seeded onto glass coverslips and allowed to attach for approximately 24 hours. Cells were washed with PBS and fixed in 3% paraformaldehyde and 2% sucrose for 15 minutes at room temperature. Fixed cells were washed in PBS and quenched in 0.1% glycine for 5 minutes at room temperature. Cells were then permeabilized in 0.1% triton for 15 minutes at room temperature, washed in PBS and exposed to blocking buffer (50%FBS in PBS) for 15 minutes at room temperature. Cells were incubated in Primary antibody (1:1000) in 10% FBS PBS overnight at 4° C. Cells were then washed 3 times in PBS, and incubated in secondary antibody (Alexa Fluor 488 goat

anti-rabbit IgG (1:500 in 10% FBS in PBS)), washed an additional 3 times and coverslips were mounted onto slides with vectorshield and viewed using a Zeiss Axiovert 200 microscope.

Proliferation and apoptosis assays. Apoptotic assays were performed as previously described (34). For breast cancer cell lines, apoptotic assays were performed on whole cell population and not gated cells. To determine G1/S ratios, cells were co-transfected with GFP and HA-PHLPP2 and incubated with high serum (10% FBS) for 48 hrs; cells were gated based on GFP expression as described previously (34). For BrdU incorporation assays, cells were maintained in high growth media (10% FBS) and transfected with 100nM smartpool siRNA and incubated for 48hrs prior to performing assays following manufacturer's protocol (Oncogene Research Products).

Statistical Analysis. Statistical analysis was performed using JMP 5.1 statistical software (SAS Institute Inc., Cary, North Carolina, United States). The significance of differences between siRNA control and siPHLPP1, siPHLPP2 or both was determined using one-way ANOVA statistical analysis followed by post-hoc Student's t tests.

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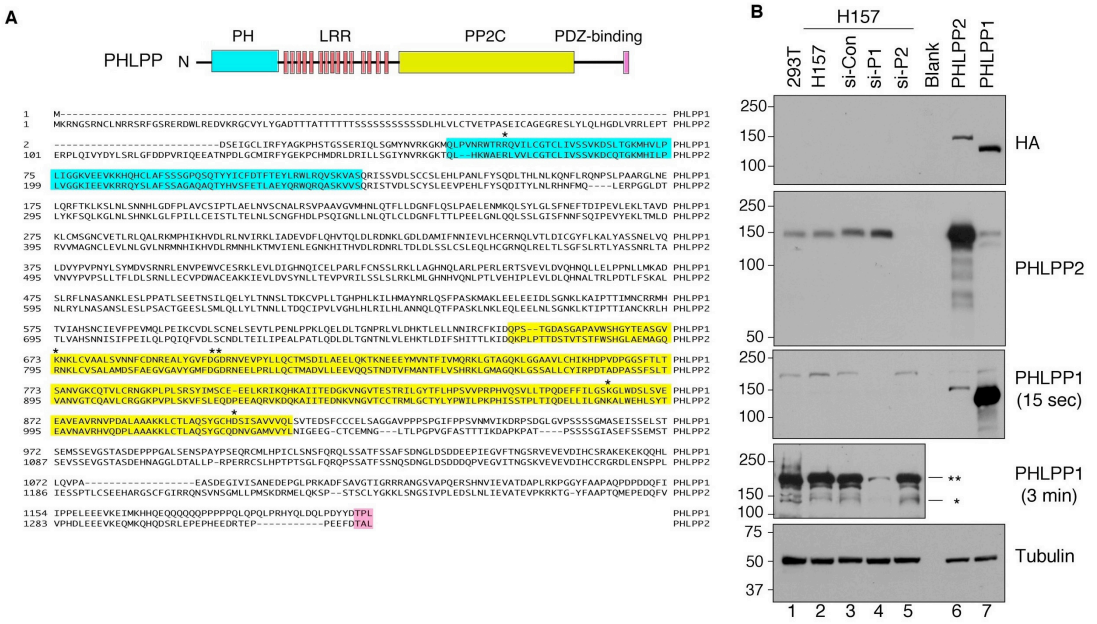

Figure 2.1: Domain composition and characterization of PHLPP2. (A), Domain structure and sequence alignment of PHLPP1 and PHLPP2 showing PH domain (cyan), leucine-rich repeat (red), phosphatase domain (yellow), and PDZ-binding motif (pink). Asterisks indicate conserved key residues within PH and PP2C domains described in Gao et al., 2005. (B), Western blot of lysates from 293T (lane 1) or H157 NSCLC (lane 2) cells probed with isoform-specific antibodies for PHLPP1 or PHLPP2; two exposures shown for PHLPP1 blot. Asterisk denotes PHLPP1a and double asterisk denotes PHLPP1b. For migration controls, H157 lysates transfected with HA-PHLPP2 (lane 6) or HA-PHLPP1 (lane 7) were analyzed with HA antibody. H157 cells were treated with non-targeting siRNA controls (si-Con; lane 3), smartpool siRNA for PHLPP1 (si-P1 lane 4), or smartpool siRNA for PHLPP2 (si-P2; lane 5).

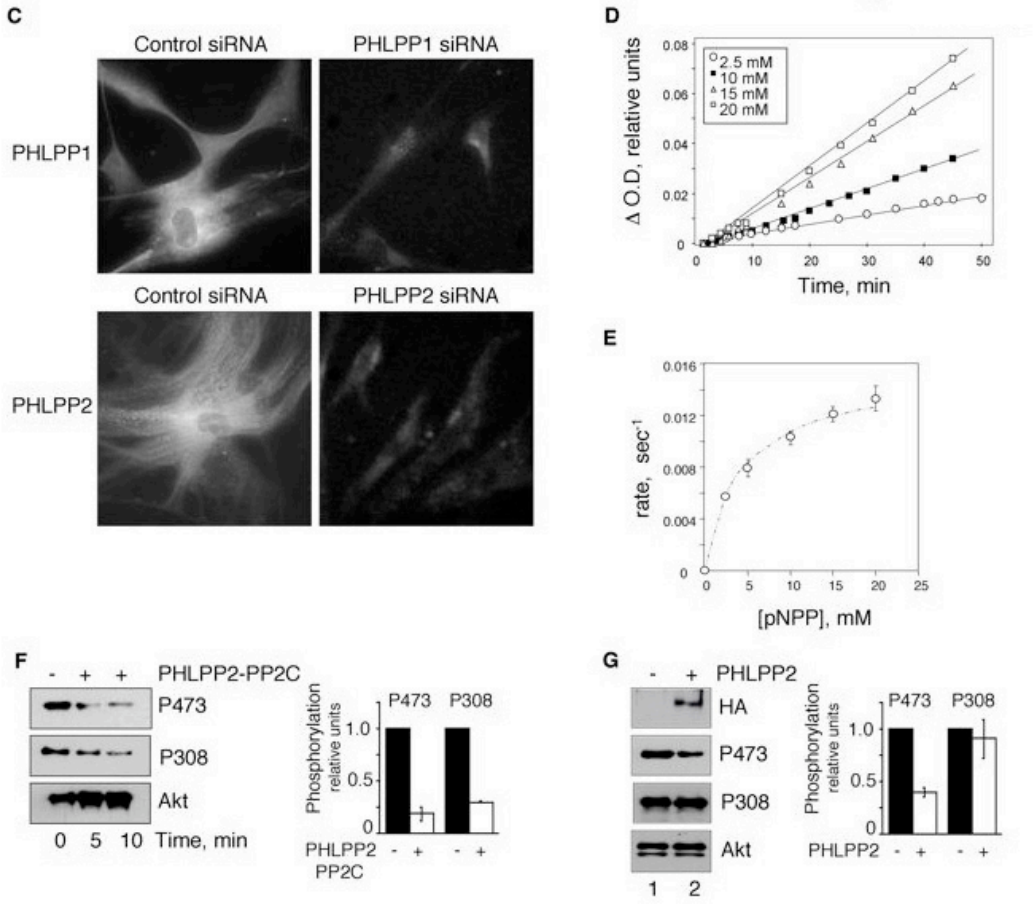

Figure 2.1: (C) Hs578Bst cells treated with control siRNA or isoform-specific PHLPP siRNA were stained using PHLPP1-specific (upper panel) or PHLPP2-specific (lower panel) antibodies and analyzed by immunofluorescence. (D) Bacterially-expressed GST-PP2C domain of PHLPP2 (100 nM) was incubated with the indicated concentrations of *p*-nitrophenylphosphate (*p*NPP) and the dephosphorylation continuously monitored by detecting the change in absorbance at 405 nm, as described in Methods. Dephosphorylation was linear with time under the conditions of this assay up to 10 hr. (E) Initial rates of *p*NPP dephosphorylation were plotted as a function of substrate concentration. Data represent the mean ± SEM of at least three independent experiments. (F) Dephosphorylation of pure His-tagged Akt1 was detected following incubation with purified PHLPP2-PP2C domain for 5 (lane 2) or 10 (lane 3) min; PHLPP2-PP2C domain was omitted in lane 1. Quantification of three independent experiments showing relative phosphorylation of Akt at P308 and P473 at the 5 min time point; error bars indicate standard deviation. (G) 293T cells were transfected with vector (lane 1) or HA-PHLPP2 (lane 2) under high serum conditions (10% FBS DMEM); thereafter HA-PHLPP2 was immunoprecipitated and incubated with pure phosphorylated Akt1 for 10 minutes. Akt phosphorylation was detected using phospho-specific antibodies. Bar graph summarizes data from three independent experiments; error bars indicate standard deviation.

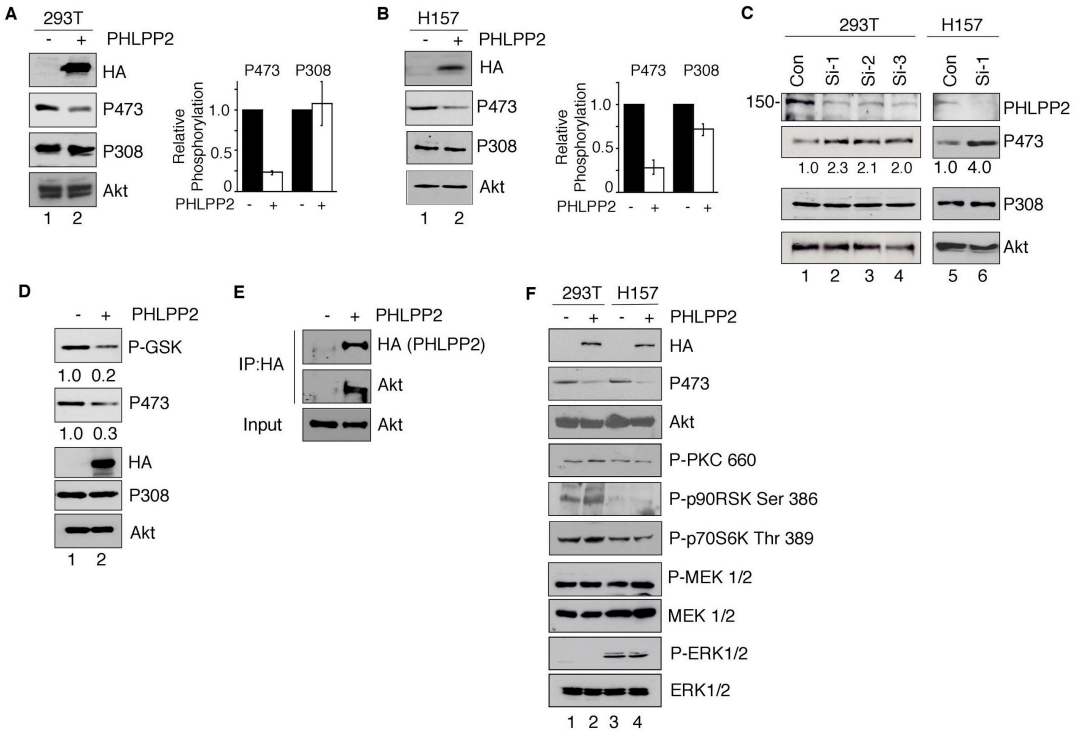

Figure 2.2: *In vivo* characterization of PHLPP2. **(**A,B) 293T and H157 cells were transfected with vector (lane 1) or HA-PHLPP2 (lane 2) for 48 hr under high serum conditions prior to lysis. The phosphorylation of Akt in lysates was detected by Western blot analysis. Data from three independent experiments are summarized in bar graph (relative phosphorylation of Akt at P473 or P308 was normalized to total Akt). Error bars indicate standard deviation. (C) Cell lysates from 293T or H157 cells transfected with control (Con) or PHLPP2-specific siRNA (Si-1, Si-2, Si-3) for 48 hr under high serum conditions. The phosphorylation of Akt and relative protein levels of Akt and PHLPP2 were detected by Western blot analysis using PHLPP2, phospho-specific Akt and total Akt antibodies. Relative S473 phosphorylation, normalized to total Akt, is indicated below the blot. Blots are representative of three independent experiments. (D) Akt was immunoprecipitated from 293T cells transfected with vector (lane 1) or HA-PHLPP2 (lane 2) for 48 hr under high serum conditions and incubated with a GSK-3-fusion protein in an *in vitro* kinase assay. GSK-3 phosphorylation was assessed by Western blot analysis with phospho-specific GSK-3 antibodies. The relative phosphorylation was normalized to total Akt and quantified below the blots. (E) HA-PHLPP2 was immunoprecipitated from 293T cells transfected with vector (lane 1) or HA-PHLPP2 (lane 2). Immunoprecipitates were subsequently analyzed by Western blot analysis for the presence of Akt or PHLPP2. (F) 293T and H157 cells were transfected with vector (lane 1) or HA-PHLPP2 (lane 2) for 48 hr under high serum conditions. The phosphorylation of Akt, ERK 1/2, MEK 1/2, PKC, p70S6K, and p90RSK in lysates was detected by Western blot analysis; blot is representative of three independent experiments.

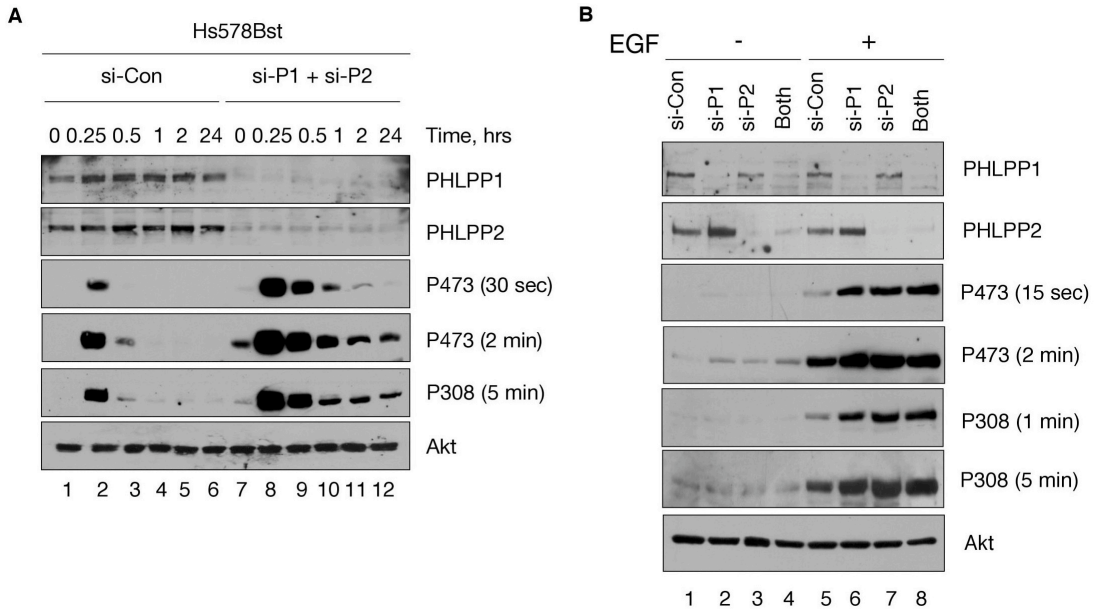

Figure 2.3: PHLPP isoforms control the amplitude of agonist-stimulated Akt phosphorylation. (A) Hs578Bst cells, a normal breast cell line, were transfected with smartpool siRNA to both PHLPP1 and PHLPP2 under high serum conditions and incubated for 48 hr. Media was then changed to low serum (0.1% FBS DMEM) and cells incubated 2 hours prior to addition of EGF (10 ng/ml) for 24 hr timepoint or overnight for all other EGF timepoints. The phosphorylation of Akt and protein levels of Akt, PHLPP1 and PHLPP2 in lysates were detected by Western blot analysis; exposure times indicated for phosphoblots. (B) Hs578Bst cells were transfected with smartpool siRNA to PHLPP1, PHLPP2 or both under high serum conditions and incubated for 48 hr. Media was then changed to low serum and cells cultured overnight prior to addition of EGF (10 ng/ml) for 15 min. The phosphorylation of Akt and protein levels of Akt, PHLPP1 and PHLPP2 in lysates were detected by Western blot analysis.

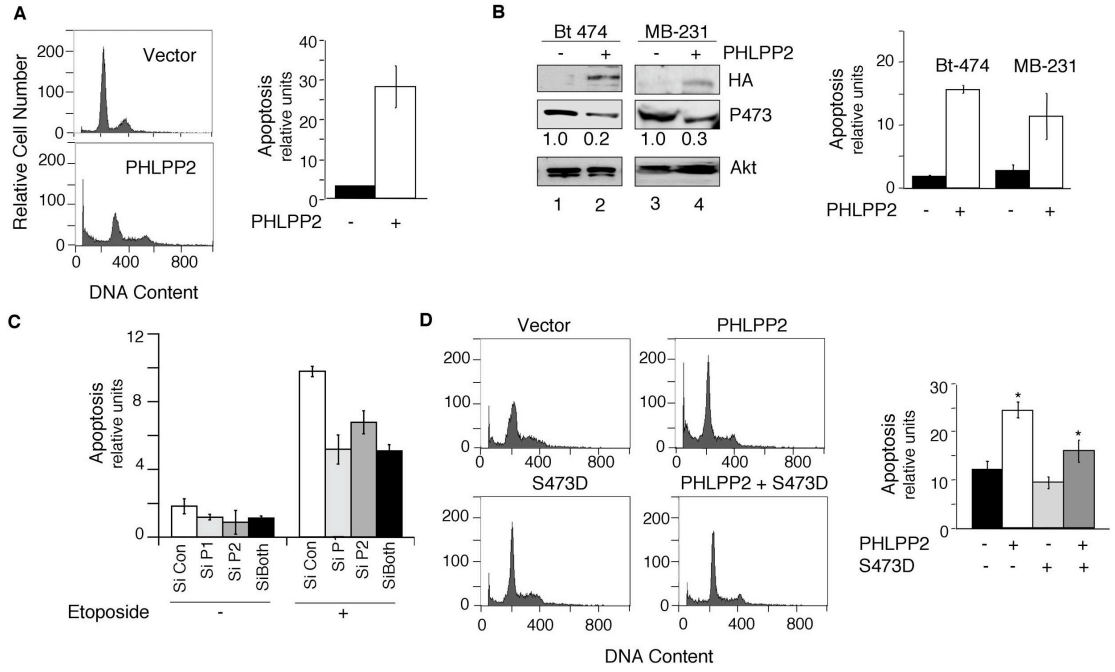

Figure 2.4: PHLPP2 regulates apoptosis and the cell cycle. (A) H157 NSCLC cells were transfected with HA-PHLPP2 or vector, under low serum conditions (0.1% FBS DMEM) for 48 hr and apoptosis (sub-2N DNA content) was assessed using propidium iodide incorporation assays and flow cytometry (B) HA-PHLPP2 or vector alone was expressed in breast cancer cell lines for 48 hr under low serum conditions and the phosphorylation of Akt in lysates was detected by Western blot analysis. Relative phosphorylation is normalized to total Akt. Apoptosis was assessed using propidium iodide incorporation assays and flow cytometry in cells expressing HA-PHLPP2 or vector alone. (C) H157 cells were transfected with smartpool siRNA to PHLPP1, PHLPP2 or both under high serum conditions and incubated for 48 hr. Media was then changed to low serum conditions, etoposide (50 mM) was added for 24hr, and apoptosis was measured by flow cytometry. (D) Expression of Akt S473D rescues PHLPP2-induced apoptosis. MDA-MB-231 cells were transfected with indicated constructs for 48 hr under low serum conditions. Histograms show sub-2N DNA; quantitation of sub-2N DNA is indicated in bar graph. One-Way ANOVA with post-hoc student's t test was performed on data from S473D and PHLPP2 transfections and compared to empty vector control; asterisks indicate $p < 0.01$.

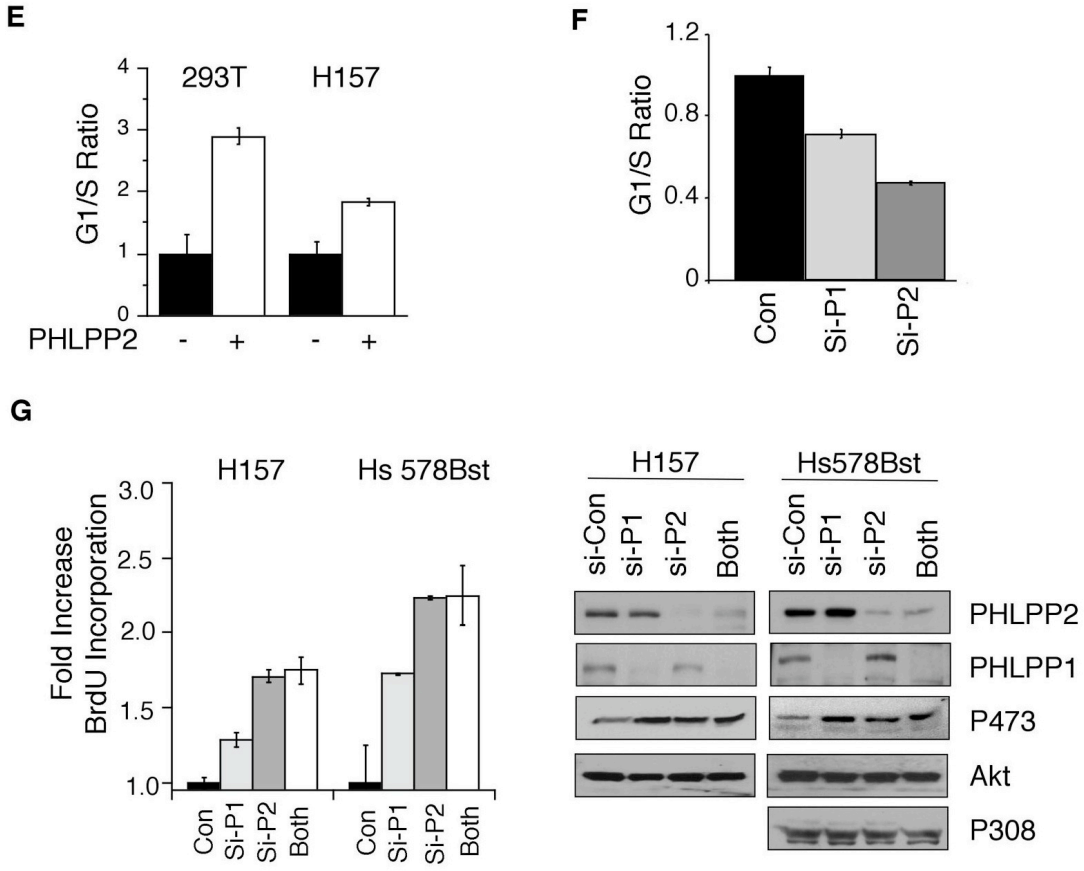

Figure 2.4: E, An increase was observed in the G1/S ratio in cells transfected with HA-PHLPP2 compared to cells transfected with vector alone. Cells were transfected for 48 hr under high serum conditions, and the G1/S ratios were determined by propidium iodide incorporation assays and flow cytometry**.** (F) Knock-down of PHLPP1 (Si-P1) or PHLPP2 (Si-P2) for 48 hr under high serum conditions decreased G1/S ratio in H157 cells as assessed by flow cytometry. (G) Knock-down of PHLPP1, PHLPP2, or both for 48 hr under high serum conditions increased BrdU incorporation in H157 and Hs578Bst cells. Western blots were performed in parallel to ensure the PHLPP phosphatases were being sufficiently knocked down. For all panels in figure 4 data in bar graphs are representative of assays performed in triplicate, with error bars indicating standard deviation, and are representative of three independent experiments.

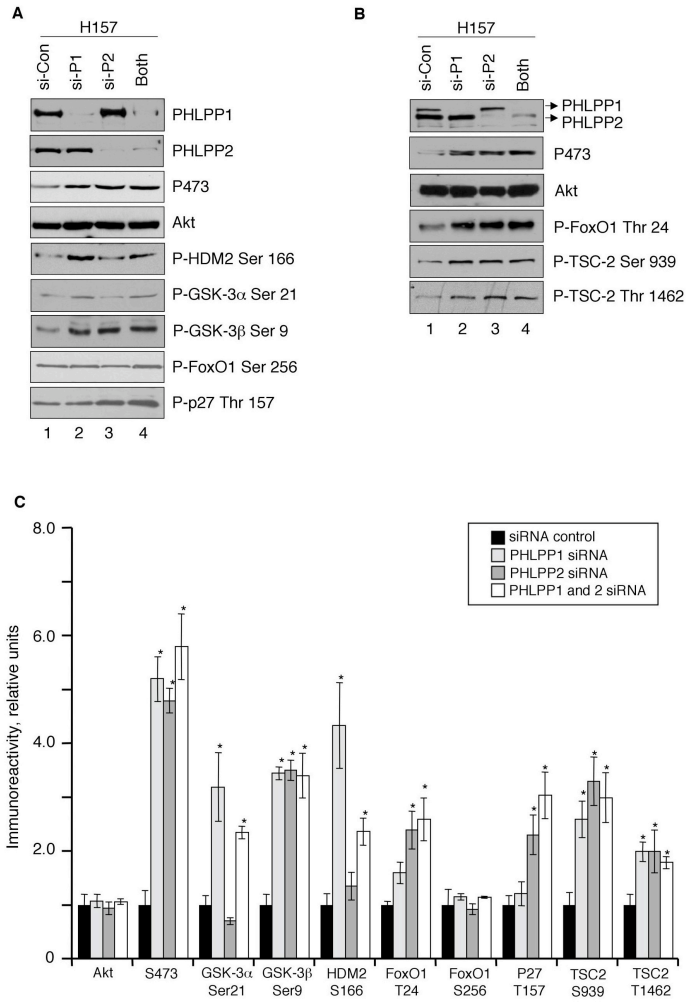

Figure 2.5: PHLPP1 and PHLPP 2 differentially regulate Akt downstream substrates. (A) H157 cells were transfected with non-targeting siRNA control (si-Con), PHLPP1 smartpool siRNA (si-P1), PHLPP2 smartpool siRNA (si-P2), or smartpool siRNA for both PHLPP1 and PHLPP2 (Both) for 48 hr under high serum conditions and lysates were analyzed by Western blot. The phosphorylation state of Akt, HDM2, GSK-3a/b, FoxO1, p27 and relative protein levels of Akt, PHLPP1, and PHLPP2 were detected using the indicated phospho-specific and total endogenous protein antibodies. Western blots are representative of three independent experiments. (B) As in A, H157 cells were transfected with smartpool siRNA to PHLPP1, PHLPP2, or both, incubated 48 hr under high serum conditions and lysates were analyzed by Western blot. The phosphorylation state of FoxO1, Akt, and TSC2 and relative protein levels of Akt, PHLPP1 and PHLPP2 were detected using indicated antibodies. Western blots are representative of three independent experiments. (C) Quantification of phosphorylation of immunoreactivity of three independent experiments as in A and B. One-Way ANOVA with post-hoc student's t test was performed on all data from siRNA treatments compared to control; asterisks indicate $p < 0.05$. Error bars indicate standard error of the mean from three independent experiments.

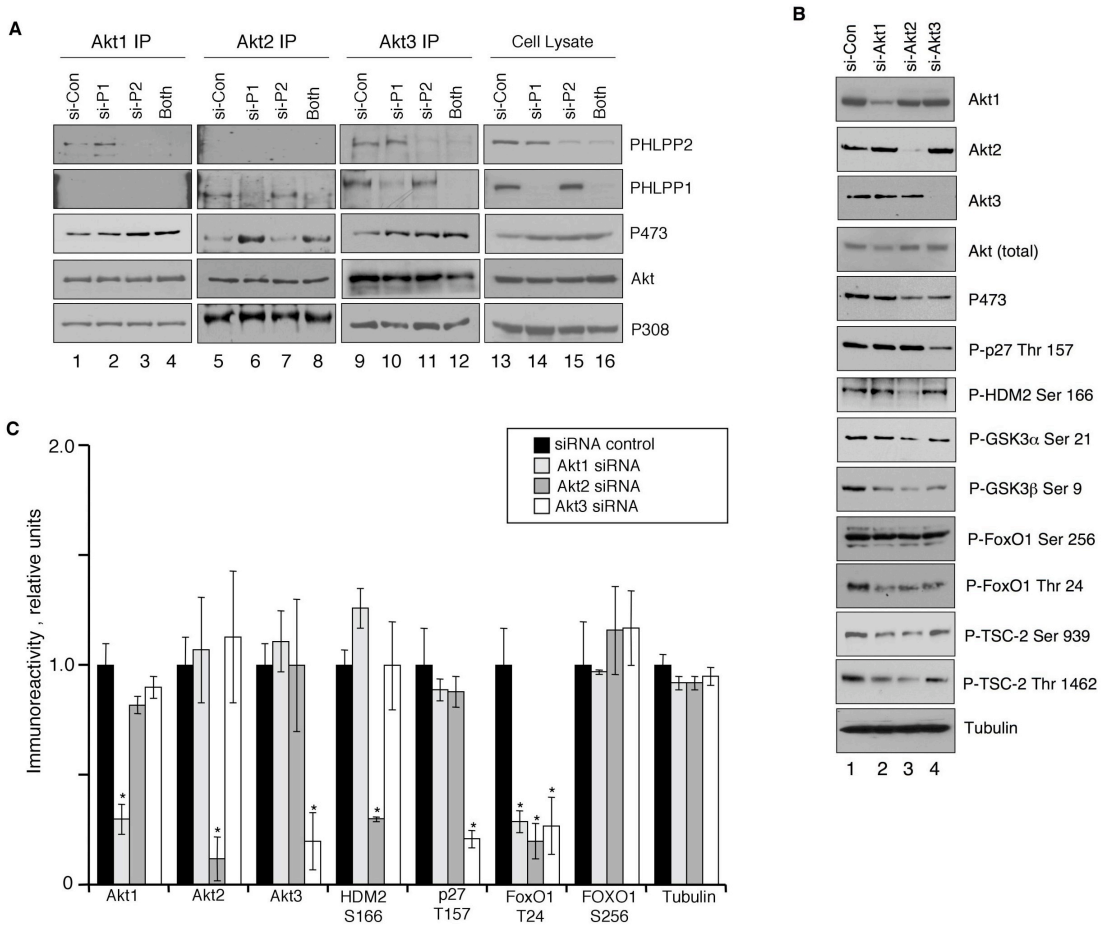

Figure 2.6: PHLPP 1 and PHLPP2 regulate specific Akt isoforms. (A) Akt isoforms were immunoprecipitated from H157 cells transfected with non-targeting control siRNA (si-Con), PHLPP1 smartpool siRNA (si-P1), PHLPP2 smartpool siRNA (si-P2) or siRNA for both PHLPP1 and PHLPP2 (Both) for 48 hr under high serum conditions. Immunoprecipitates were analyzed by Western blot analysis for the presence of Akt, Akt phosphorylation on Ser473 (P473) or Thr308 (P308), PHLPP1 or PHLPP2. (B) Akt isoforms were knocked down with Akt isoform-specific siRNA for 48 hr under high serum conditions and cell lysates were analyzed by Western blot with the following antibodies: Akt isoform-specific antibodies, Akt pan antibody (Akt total), Ser473 antibody (P473) and phospho-specific antibodies to indicated downstream substrates. a-tubulin was used as a loading control. Western blots are representative of three independent experiments. (C) Quantification of immunoreactivity of indicated bands from 3 independent experiments as described in B. One-Way ANOVA with post-hoc student's t test was performed on data from all siRNA treatments compared to control; asterisks indicate $p < 0.05$. Error bars indicate standard error of the mean.

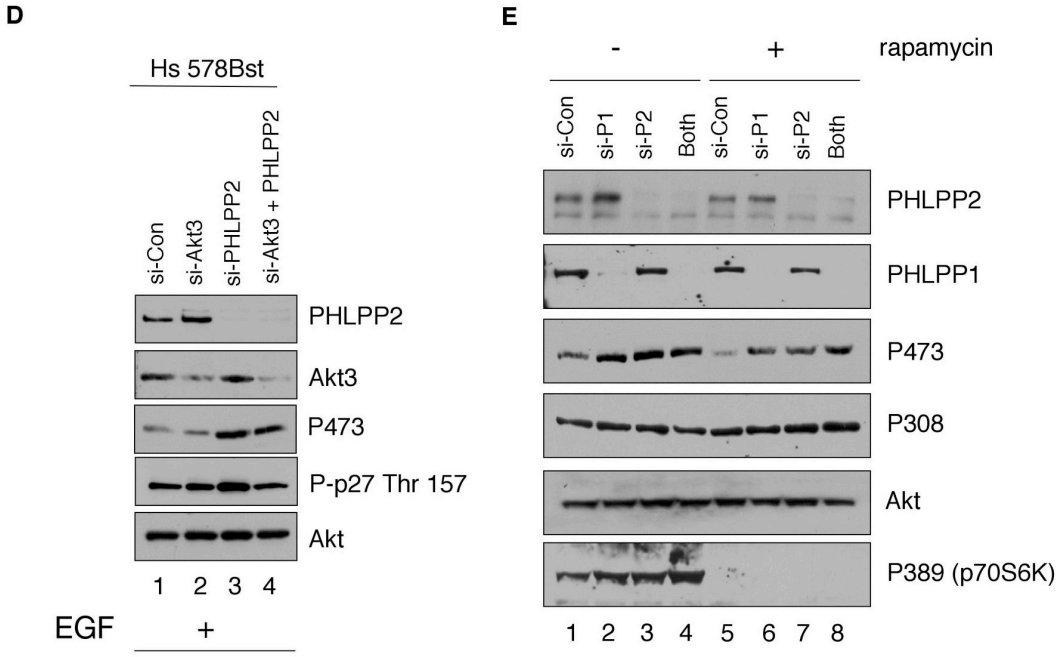

Figure 2.6: (D) Hs578Bst cells were transfected with siRNA to Akt3, PHLPP2 or both, incubated 48 hr under high serum conditions. Media was changed to low serum overnight and EGF (10 ng/ml) was added for 15 min prior to cell lysis. Lysates were assessed by Western blot analysis for phospho-Akt, phospho-p27, Akt, Akt3 and PHLPP2. (E) Rapamycin treatment unmasks additivity in PHLPP1 and PHLPP2 knock-down experiments. H157 cells were transfected with smartpool siRNA to PHLPP1, PHLPP2 or both, incubated for 48 hr under high serum conditions and then treated with rapamycin (100 nM) for 24 hr. Lysates were analyzed by Western blotting using phospho-Akt, phospho-p70S6K, Akt, PHLPP1 and PHLPP2. Western blot is representative of three independent experiments.

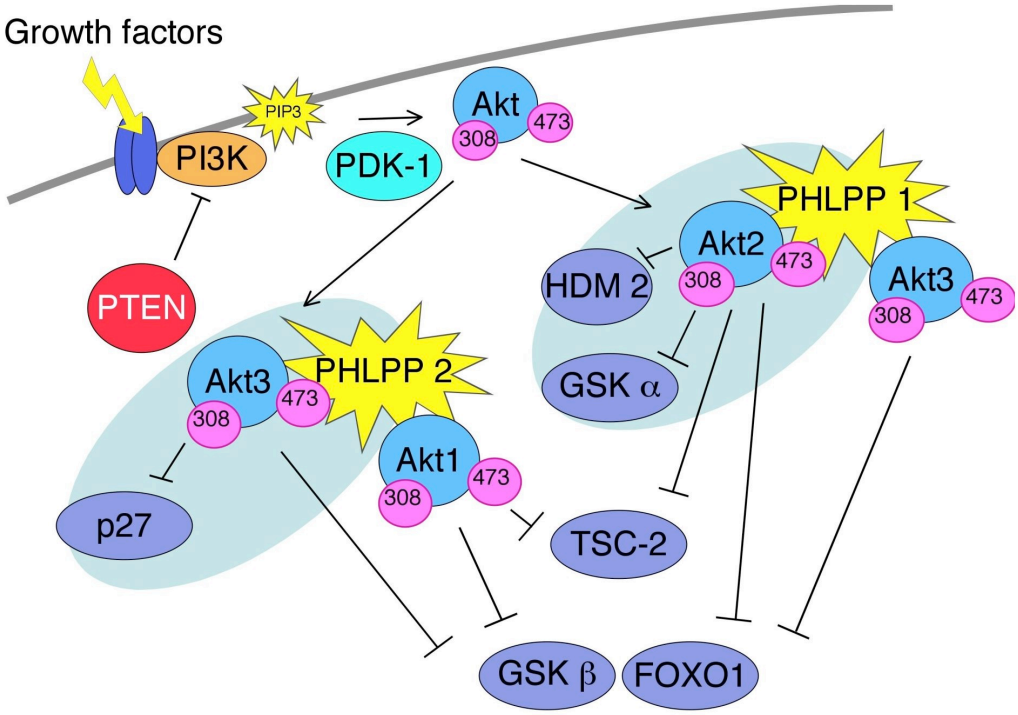

Figure 2.7: Model illustrating how specificity in signal termination by PHLPP isoforms is achieved by specific PHLPP-Akt-substrate complexes. Akt is activated following receptor-mediated activation of PI3K to generate PtdIns 3P (PIP_3). This second messenger recruits Akt to the plasma membrane, where it is phosphorylated by PDK-1 at the activation loop (Thr 308) an event that triggers phosphorylation at the hydrophobic motif, (Ser 473). Akt then redistributes to specific intracellular locations, presumably in complex with specific substrates. Signal termination is achieved at the initial step by the lipid phosphatase PTEN, which removes the activating lipid, or, once signaling has been initiated, by the protein phosphatase PHLPP, which dephosphorylates Ser 473 on Akt. Specific complexes (blue shaded ovals) of PHLPP1 or PHLPP2 with Akt1, Akt2, of Akt3 allow PHLPP isoforms to differentially terminate Akt signaling. For example, compartmentalization of PHLPP2-Akt3-p27 (blue shaded oval on left) and PHLPP1-Akt2-HDM2/GSK-3α (blue shaded oval on right) define unique pathways in the Akt signaling network. Some substrates, such as FoxO1, GSK-3β and TSC2 are inactivated by both PHLPP isoforms via regulation of all 3 Akt isoforms.

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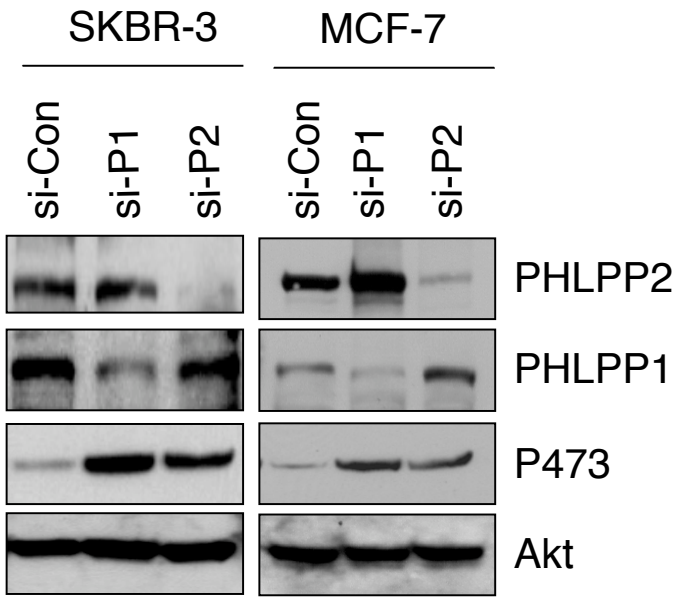
Supplemental Data

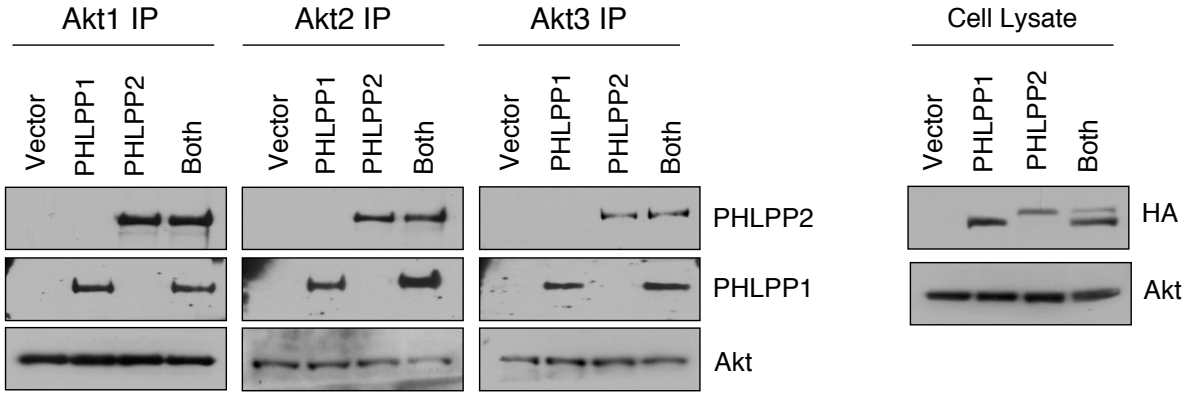
Materials and Methods. The following antibodies were purchased from Cell Signaling: phospho-specific to Thr308 (P308) and Ser473 (P473) of Akt, Akt, isoform-specific to Akt1, Akt2 and Akt3, phospho-specific to GSK-3α/β, p70S6K, p90RSK, TSC2, FoxO, ERK 1/2, MEK 1/2, and total MEK 1/2 and ERK 1/2. Phospho-specific antibody to p27 was purchased from R&D systems. A PHLPP2-specific antibody was generated using the following peptide: RGSGFGIRRQNSYNS as previously described (Gao et al., 2005). Additionally, PHLPP1 and PHLPP2 isoform-specific antibodies became available during the course of these studies and were purchased from Bethyl Laboratories. All experiments used the Bethyl Laboratory antibodies unless otherwise indicated in figure legends. EGF and the Akt agarose used for immunoprecipitations was purchased from Upstate Biotechnologies. Rapamycin was purchased from Calbiochem. 6xHis-tagged human Akt1 was purified from baculoviral-infected Sf21 cells as described previously (Gao et al., 2005). Bt-474 breast cancer cells and Hs578Bst normal breast cells were purchased from the ATCC. All other chemicals were reagent-grade.

Supplemental Experimental Procedures. HA-PHLPP1, HA-PHLPP2 or both were expressed in H157 cells under high serum conditions for 48 hr prior to immunopreciptation. H157 cells were lysed in Buffer 3 (50 mM Na_2HPO_4, pH 7.5, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton X-

100, 1 mM DTT, 200 μM benzamidine, 40μg ml^{-1} leupeptin, and 1 mM PMSF) and detergent soluble lysates were incubated overnight at 4° C with Akt isoform-specific antibodies (Cell Signaling) and ultra-link protein A/G beads (Pierce). Immunoprecipitates were washed four times in Buffer 3, and probed with Akt, PHLPP1 or PHLPP2 specific antibodies from Cell Signaling and Bethyl Laboratories.



Supplemental Figure 2.1: PHLPP1 or PHLPP2 regulate Ser473 phosphorylation in breast cancer cells. MCF-7 and SKBR-3 cells were transfected with non-targeting siRNA control (si-Con), PHLPP1 smartpool siRNA (si-P1), or PHLPP2 smartpool siRNA (si-P2) for 48 hrs. under high serum conditions and lysates were analyzed by Western blot. The phosphorylation state of Akt and relative protein levels of Akt, PHLPP1, and PHLPP2 were detected using the phospho-specific Akt and total endogenous protein antibodies. Western blots are representative of three independent experiments.



Supplemental Figure 2.2: Overexpressed PHLPP1 and PHLPP2 co-immunoprecipitates with all three Akt isoforms. Akt isoforms were immunoprecipitated from H157 cells transfected with vector (lane 1), HA-PHLPP1 (lane 2), HA-PHLPP2 (lane 3) or both (lane 4) for 48 hrs. under high serum conditions. Immunoprecipitates were analyzed by Western blot analysis for the presence of Akt, Akt phosphorylation on Ser473 (P473), PHLPP1, or PHLPP2.

This Chapter is, in full, a reprint of the material as it appears in Molecular Cell, 2007, Brognard J, Sierecki E, Gao T, Newton AC. I was primary research and author; co-authors provided technical assistance and helped direct and supervise the research included in this chapter. The supplementary data is, in full, a reprint as it appears online at www.molecule.org/cgi/content/full/25/6/917/DC1.

Chapter 3

Identification and Functional Characterization of a Novel PHLPP2 Variant in Breast Cancer: Impacts on Akt and PKC Phosphorylation

Abstract

The recently discovered PHLPP2 (PH domain Leucine-rich repeat Protein Phosphatase 2) decreases Akt (PKB) activity by specifically dephosphorylating the kinase at a key regulatory site, the hydrophobic phosphorylation motif (Ser473 in Akt1). PHLPP2 is poised to be a tumor suppressor based on its negative regulation of Akt as well its chromosomal location at 16q22.3, a region that encounters frequent loss of heterozygosity in breast cancer. We have identified a polymorphism that results in an amino acid change from a Leu to Ser at codon 1016 in the phosphatase domain of PHLPP2. Analysis of a HA-PHLPP2 construct with a Ser at position 1016 revealed reduced phosphatase activity towards phosphorylated Akt both *in vitro* and in cells compared to HA-PHLPP2 with a Leu at position 1016. Consistent with this reduced activity, induction of apoptosis in cells expressing the Ser1016 variant was reduced 2.5-fold compared to cells expressing the Leu1016 variant. Depletion of endogenous PHLPP2 variants in breast cancer cells revealed that basal Akt phosphorylation and total protein kinase C protein levels were increased in cells depleted of the Leu1016 variant, but not in cells depleted of the Ser1016 variant. Sequencing of pair-matched normal and tumor tissues from the same patients revealed that the Ser allele is preferentially retained in high-grade breast tumors, where we observe loss of heterozygosity; similarly we observed retention of the only the Ser allele in the breast cancer cell line (Hs578t) compared to its normal heterozygous counterpart (Hs578Bst). We investigated whether the Ser1016 variant was over-represented in women with breast cancer in a US population-based case-control study

of Caucasian women (714 cases, 583 controls). Approximately 15% of controls carried at least one Ser allele. Carriers were at no greater risk of breast cancer than noncarriers (age- and study site-adjusted odds ratio (OR): 1.01; 95% confidence interval (CI): 0.79-1.30; p=0.93). Women homozygous for the Ser1016 variant (2.7% of controls) had a nonsignificantly reduced breast cancer risk when compared to women homozygous for the leu allele (age- and site-adjusted OR: 0.56; 95% CI: 0.25, 1.26; p=0.16) though this result was based on sparse data. In conclusion, we have identified a functional polymorphism that impairs the activity of PHLPP2 though its role in human breast tumorigenesis is complex and remains to be determined.

Introduction

Breast cancer is diagnosed in approximately 180,000 women and is the cause of 40,000 deaths each year in the US (1). A prevalent underlying mechanism driving tumorigenesis is aberrant signal transduction pathways that result in constitutive activation of cell growth/proliferation and/or survival pathways (2). A well-characterized signal transduction pathway in breast cancer that promotes both cellular survival and growth/proliferation is the PI3K/Akt pathway (3). This pathway is activated by a number of mechanisms, including amplification or gain of function mutations in upstream receptor protein tyrosine kinases (RPTKs) (4, 5), constitutive activation of hormone receptors (6) , activating mutations in PI3K and Akt (7, 8), and loss of function mutations in the regulatory phosphatase PTEN (phosphatase and tensin homolog on chromosome ten) (9). Thus, Akt is a major regulator of breast tumorigenesis.

There are three isoforms of Akt present in humans, Akt1, Akt2, and Akt3. All three isoforms contain activating phosphorylation sites in the activation loop (Thr308 in Akt1) and in the C-terminus hydrophobic motif (Ser 473 in Akt1) (10). Upon growth factor receptor stimulation, PI3K becomes activated and phosphorylates the D3 position of, typically, phosphatidylinositol (4,5) bisphosphate to generate phosphatidylinositol (3,4,5) trisphosphate (PIP3) (11). This 3'-phosphorylated lipid recruits Akt to the plasma membrane by binding to its PH domain, resulting in conformational changes that allows access to the activation loop phosphorylation site (11). Constitutively bound PDK-1 (phosphatidylinositol dependent kinase-1) then

phosphorylates Akt at Thr 308, accompanied by phosphorylation at Ser 473 resulting in a catalytically-active kinase that phosphorylates downstream substrates in the cytoplasm and nucleus (12). Phosphorylation of Ser473 depends on the mTORC2 complex containing mTOR, rictor, mLST8, and mSin1 variants (13-17), however whether by directly phosphorylating this site or by regulating events that allow phosphorylation (possibly autophosphorylation (13)) at this site, remains to be clarified. Signalling through this pathway is terminated by removal of the lipid second messenger PIP3 catalyzed by the phosphatase PTEN and by direct dephosphorylation of Akt by the PHLPP family of phosphatases and PP2A type phosphatases (18-21).

The role of each Akt isoform in breast cancer initiation and progression is complex and is likely dependent on varying genetic backgrounds. Akt1 inhibits both invasion and cellular migration in breast cancer cell lines. It also inhibits migration and the phenotypic hallmarks of the epithelial-mesenchymal transition in the immortalized MCF-10A cells engineered to overexpress the IGF-I receptor (22, 23). Akt1 inhibits migration and invasion through inhibition of the transcription factor NFAT, enhanced ERK signaling, and regulation of the tumor suppressor TSC-2 (22-24). Conversely, Akt1 has also been reported to promote tumorigenesis and metastasis in mice expressing ErbB2 as well as promoting migration in mouse endothelial tumor cells (25). Consistent with the latter result, Akt1 knockout mice have delayed tumor onset when crossed with mice expressing the oncogenes ErbB2 or polyoma middle T antigen (26). A recent study has also reported the identification of a somatic mutation in the PH domain of Akt1 in 8% of breast cancers leading to activation of the kinase

(8). These studies highlight the complexity of Akt1's role in breast cancer and provide evidence that Akt1 can be both a tumor suppressor and an oncogene. Akt2 activation has been observed in 40% of primary breast tumors and activation of Akt2 leads to increased migration, invasion and metastasis in cell culture systems (6, 23, 27, 28). Surprisingly Akt2 knockout mice crossed with mice expressing ErbB2 or polyoma middle T antigen have an increased rate of tumor development, suggesting Akt2 may inhibit tumor progression (26). Finally, the expression of Akt3 has been reported to be elevated in ER negative breast cancer cell lines, an event which may promote resistance to chemotherapy (29, 30). These observations highlight the complexity in understanding the contribution of each Akt isoform in the process of tumorigenesis.

Our lab has recently identified a novel family of phosphatases termed PHLPP. The two isoforms PHLPP1 and PHLPP2 negatively regulate Akt by dephosphorylating the hydrophobic motif, an event that inhibits Akt activity and promotes apoptosis (19, 20). PHLPP2 binds and dephosphorylates Akt1 and Akt3 whereas PHLPP1 regulates Akt2 and Akt3 (19, 31). Given the complexity surrounding the role of Akt1 in breast cancer we hypothesized that PHLPP2 could potentially be both a tumor suppressor and an oncogene and this would be dependent upon on the genetic makeup of individual patients.

Supporting the role of PHLPP2 as a potential tumor suppressor, we have recently discovered that the PHLPP phosphatases dephosphorylated the hydrophobic motif of PKC, resulting in degradation of PKC. Depletion of the PHLPP phosphatases results in an increase in PKC phosphorylation at the hydrophobic motif (Ser657

PKCα) and increased PKC protein levels (T. Gao, J. Brognard, A. Newton, unpublished results). PKC is well-characterized oncogene and loss of function of proteins that would increase PKC protein levels could promote tumorigenesis (32).

Providing further rationale that PHLPP2 could be a potential tumor suppressor, the phosphatase is located on chromosome 16q22.3, a region that encounters frequent loss of heterozygosity in many primary and malignant breast tumors (33). In lobular breast carcinomas the tumor suppressor gene in this region has been identified as the E-cadherin protein (CDH1 gene), however the tumor suppressor genes (TSG) in ductal carcinomas remain elusive, and E-cadherin is not mutated in ductal carcinomas (33, 34).

Here we identify a nonsynonymous polymorphism that results in an amino acid change from a Leu to a Ser at codon 1016 in the PP2C domain of the PHLPP2 phosphatase. Overexpression studies reveal the Ser1016 variant has impaired phosphatase activity and is less effective at inducing apoptosis than the Leu1016 variant. When comparing a pair-matched normal and breast cancer cell line or pair-matched normal and high-grade tumor patient samples that are heterozygous, we observe loss of the Leu allele. This provides evidence that PHLPP2 could be one of the elusive tumor suppressor genes on chromosome 16q and loss of the more catalytically active Leu1016 may promote breast tumorigenesis. However, the Ser1016 genotype was not more common among women affected by breast cancer in a US case-control study; unexpectedly, women homozygous for the Ser allele had a nonsignificantly decreased breast cancer risk. Although the functional consequences

of the L1016S PHLPP2 variant seem unequivocal based on these results, further studies are needed to determine its relationship with breast cancer risk.

Materials and Methods

Materials. Smartpool siRNA against PHLPP1 and PHLPP2 was purchased from Dharmacon. The following antibodies were purchased from Cell Signaling: phospho-specific to Thr308 and Ser473 of Akt, Akt, PTEN, and ERK1/2. PHLPP1 and PHLPP2 isoform-specific antibodies were purchased from Bethyl Laboratories. PKCα, Annexin I, and Lamin A antibodies were purchased from Santa Cruz Biotechnology. EGF was purchased from Upstate Biotechnologies. 6xHis-tagged human Akt1 was purified from baculoviral-infected Sf21 cells as described previously (20). Breast cancer cell lines or DNA from the cell lines listed in Figure 1B were purchased from the ATCC. Breast tumor tissue DNA was purchased from Asterand or provided by Dr. Linda Wasserman. Asterand provided histology for tumor tissue samples. 100 DNA samples extracted from tissues of healthy individuals were purchased from the Coriell Institute. All other chemicals were reagent-grade.

Cell culture, transfections, and immunoblotting. ZR-75-1, T47D, MDA-MB-468, and SKBR3 cell lines were maintained in RPMI 1640 (Cellgro), and all other cell lines were maintained in DMEM (Cellgro); both media were supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained at 37ºC in 5% CO_2. Transient transfections and siRNA experiments were performed as previously described (19). Briefly, transient transfections were performed using Effetene

reagents (Qiagen) following the manufacturers protocol. Cells were plated in six-well plate at $2x10^5$ cells per well and incubated overnight, prior to the addition of transfection reagents containing 0.5 μg DNA. Transfected cells were incubated 48 hr. prior to lysis. Transfection efficiencies for 293T and H157 cell lines ranged from 70-90%. SiRNA transfections were performed using lipofectamine 2000. Briefly, cells were plated at $2x10^5$ cells per well, incubated overnight and transfected with 50 nM smartpool siRNA. The cells were incubated for 48 hr. and either lysed or their media was changed to low serum conditions for 12 hours and then EGF (10 ng/ml) was added for the indicated times prior to lysis. For immunoblotting, transfected cells were lysed in Buffer 1 (50 mM Na_2HPO_4, pH 7.5, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% SDS, 1 mM DTT, 200 μM benzamidine, 40 μg ml^{-1} Leupeptin, and 1 mM PMSF) and sonicated for 5 seconds. Lysates containing equal protein (determined by Bradford assay) were analyzed on SDS-PAGE gels, and individual blots were probed using the antibodies indicated in Figure legends. Densitometric analysis was performed with the NIH Image analysis software (version 1.63).

Phosphatase assays. The activity of full-length PHLPP2 variants was assessed by expressing the HA-PHLPP2 variants and then immunoprecipitating the PHLPP2 variants from H157 cell lysates via the HA tag. Cells were lysed in Buffer 2 (20 mM HEPES, pH 7.4, 1% Triton X-100, 1 mM DTT, 200 μM benzamidine, 40 μg ml^{-1} Leupeptin, and 1 mM PMSF). Detergent-soluble lysates were incubated overnight at 4° C with HA antibody and ultra-link protein A/G beads (Pierce). Beads were then

washed three times with Buffer 1, three times in phosphatase buffer, and then incubated in phosphatase buffer with purified phosphorylated Akt1 at 30° Celsius for the indicated times, as previously described (19).

Apoptosis assays. Apoptotic assays were performed as previously described (35). Briefly, cells were co-transfected with GFP and HA-Leu1016 or HA-Ser1016 and incubated in low Serum (0.1% FBS) media for 48 hr; cells were gated based on GFP expression as described previously and sub2n DNA was quantified (35).

*DNA and RNA Isolation***.** Genomic DNA was isolated from human tissues using QIAamp DNA mini kit (Qiagen) following the manufacturer's protocol. Tissue samples were incubated in ATL buffer (from kit) supplemented with proteinase K for 3 hrs at 56° C prior to DNA extraction. Total RNA was isolated from cell lines using RNeasy mini kit (Qiagen), following the manufacturer's protocol.

RT-PCR. One-step RT-PCR (Qiagen) was used to amplify the cDNA of full length PHLPP2 in the following cell lines: H157, 293T, MDA-MB-231, Hs578t, SKBR-3, MDA-MB-468, Bt474, T47D, MCF-7, and ZR-75-1. RT-PCR products were subcloned into pGEM-T easy vector (Promega) and the entire coding region of PHLPP2 was sequenced. Following the identification of a polymorphism at Leu1016, additional RT-PCR for all cell lines was performed by directly amplifying the region flanking the polymorphism using the following primers: 5'-gaggctcaaagggtgaagg-3' and 5'-ggcccccagcattatgct-3'. The RT-PCR products were gel purified and sequenced directly with nested primers. For sequencing genomic DNA (from Asterand, Dr. Linda Wasserman, or Corriel Institute), primers were generated to intronic and exonic sequences that flanked the polymorphism or the intronic sequences that flanked the

entire exon 17. The following primers were used: 5'-gtgaatggggtaacctgctg-3' and 5'-ctaccttgctgccattggtt-3' (flanking polymorphism) and 5'-agtggggcagtcatagtgct-3' and 5'-agttggctctcatcgttgct-3' (flanking exon 17). All PCR and RT-PCR reactions were performed twice and products were sequenced using nested primers to verify the polymorphism.

Fractionation. Cells were plated at $2x10^5$ cells per well, incubated overnight and then were transfected with non-targeting siRNA control or smartpool siRNA targeting PHLPP2 and incubated for 72 hr at 37 °C. Cells were lysed in 200 µl hypotonic buffer (50mM Na_2HPO_4, 2mM EDTA, 2mM EGTA, 1mM sodium pyrophosphate, 20mM NaF, 1 mM DTT, 200μM benzamidine, 40μg/ml leupeptin, and 1mM PMSF), passed through a 25G needle 12 times, and lysates centrifuged at 300 x g for 1 min. The supernatant (containing the cytosolic and membrane fractions) was removed for further fractionation (below). The pellet (containing the nuclear fraction) was resuspended in 200 µl membrane buffer (50mM Na_2HPO_4, 1% Triton X-100, 2mM EDTA, 2mM EGTA, 1mM sodium pyrophosphate, 20mM NaF, 1 mM DTT, 200μM benzamidine, 40μg/ml leupeptin, and 1mM PMSF), and centrifuged for 16,000 x g 15 min at 4° C. The resulting supernatant is defined as the nuclear fraction. The supernatant containing cytosolic and membrane fractions was centrifuged at 108,920 x g for 20 min. at 4ºC. The resulting supernatant is defined as the cytosolic fraction. The pellet was resuspended in 200 µl membrane buffer, and centrifuged at 108,920 x g for 20 min. at 4ºC; the resulting supernatant is the membrane fraction.

Breast cancer case-control study. To study association of the PHLPP2

variants with human cancer, we genotyped DNA collected in a US population-based breast cancer case-control study. Details of the study have been published previously (36). Briefly, all English-speaking female residents of Wisconsin, New Hampshire and Massachusetts (excluding the four counties that comprise metropolitan Boston) aged 20-74 with a new diagnosis of breast cancer reported to the state's cancer registry were eligible for this study. For comparability with controls, eligibility was limited to women with listed telephone numbers or a drivers' license verified by self-report (if <65 years of age) and/or Medicare beneficiaries (if >65 years). Control women were selected from population lists in each state and frequency matched to the cases on age (in 5 year strata). In addition to a study interview, women provided a sample of DNA (by cheek swab). The present analysis is limited to women enrolled in the parent case-control study between 1998 and 1999, with adequate DNA remaining to complete the current genotyping. A total of 714 (76%) women with a recent diagnosis of breast cancer (618 invasive breast cancers in women aged 20-69, 96 in situ breast cancer in women aged 20-74) and 583 (70%) population controls (aged 20-74) were genotyped for the PHLPP2 variant (L1016S). Women included in the present analysis had similar distributions on age and study center when compared to all women included in the original series (data not shown). All women were Caucasian, most of central European ancestry. The study was reviewed and approved by local investigational review boards at each academic center that enrolled subjects (University of Wisconsin for Wisconsin women, Harvard School of Public Health for Massachusetts women and Dartmouth Medical School for New Hampshire women). All participants provided written

informed consent.

Genotyping. A total of 100 DNA samples from a control population from the Coriell Institute (NA17201-17300) and all samples from the case-control study were genotyped using the following procedures. 1ng of DNA was added to PCR sequencing buffer (5µl reaction mix (Stratagene), 200nM of each primer, 1µl Pfu tubro DNA polymerase (stratagene), 10µl betaine, 2.5µl DMSO, 28µl water (50µl total reaction amount)) (water was added in place of betaine and DMSO for Coriell Institute samples due to the high quality of these samples). The following PCR scheme was used for each reaction: 2min. at 95 ºC, (1 min. at 94 ºC, 1 min. at 55 ºC, 1 min at 68 ºC) x 35, 10 min. at 68ºC, 4 ºC thereafter. The DNA-PCR products were sequenced directly using nested primers. The DNA primers used for PCR were 5'–gtgaatggggtaacctg–3' and 5'-aggccccccagcattatgctcat –3' and the nested primers used were 5'-ctgggctgtacatacctctacc-3' and 5'-cctcagaggtgagcagtgaa-3'.

Analysis. Multivariable logistic regression was used in the case-control study to obtain age- and state of residence-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for the L1016S PHLPP2 SNP. Two groups of overlapping controls were utilized in these analyses. For the analysis of breast carcinoma *in situ*, control women consisted of those aged 20-74 from Massachusetts and New Hampshire. For the analysis of invasive breast cancer, control women were aged 20-69 and resided in Massachusetts, New Hampshire, or Wisconsin. We tested for trend (assuming an additive influence of increasing presumptively high-risk alleles) by including in logistic regression models an ordinal term for the number of minor alleles (0, 1, 2).

Allele frequencies conformed to expectation under Hardy-Weinberg equilibrium among the controls (p=0.33).

Results

Identification of a polymorphism in PHLPP2. To determine if the PHLPP2 phosphatase is mutated in breast cancer, we sequenced RNA from breast cancer cell lines. We discovered a T->C nucleotide change at position 3047 in the open reading frame, that results in a amino acid change from Leu to a Ser at codon 1016 in the PP2C phosphatase domain. We observed that four out of 18 (22%) breast cancer cell lines possessed only the Ser allele, one breast cancer cell line possessed both the Ser and Leu alleles, and the majority of breast cancer cell lines possessed only the Leu allele (Figure 1A and 1B). To determine if this nucleotide change was a polymorphism, we genotyped 100 DNA samples from a control population from the Coriell Institute (NA17201-17300). We observed the Leu/Leu genotype in 70 individuals and the Leu/Ser genotype in 30 individuals (Figure 1C). We never observed the Ser/Ser genotype in the control population. Additionally, we genotyped 33 breast tumor tissue samples and observed the Leu/Leu genotype in 25 patients and the Leu/Ser genotype in 8 patients; again we did not observe the Ser/Ser genotype (Figure 1C). These results revealed that the T->C nucleotide change at position 3047 is a polymorphism present in 30% of the population.

The absence of the Ser/Ser genotype in the control population, but its presence in breast cancer cell lines, led us to ask whether this polymorphism could play a role in

breast tumorigenesis. One of the most common genetic lesions in breast cancer is loss of heterozygosity on chromosome 16q in the chromosomal location harboring PHLPP2 (33). To determine if the presence of only the Ser allele in breast cancer cell lines results from loss of heterozygosity, we genotyped the normal breast cell line (Hs578Bst) that is from the same patient as the breast cancer cell line Hs578t. We observed the genotype of the normal cell line to be heterozygous (Leu/Ser) therefore loss of heterozygosity (LOH) explains the presence of only the Ser allele in the Hs578t breast cancer cell line (Figure 1D). We cannot determine if loss of heterozygosity is the mechanism underlying the presence of the only Ser allele in the remaining breast cancer cell lines because no normal breast cell lines from the same patient are available for these cell lines. However, LOH at 16q, in the region including PHLPP2, has been reported for these cell lines suggesting that the patients from whom these cell lines were derived were likely heterozygous (37). Note that in the MCF-7 cell line, which possesses both alleles, we consistently observe decreased expression of the Leu allele based on sequencing, suggesting the Leu allele could be methylated (Fig. 1A).

We next asked whether Akt phosphorylation was altered in the tumor cell line expressing only the Ser allele compared to its normal cell counterpart with both alleles. Because the T47D, Hs578t, and ZR-75-1 breast cancer cell lines, which possess only the Ser allele, have hyper-phosphorylated Akt despite expressing wild type PTEN and having inactivated ErbB2 receptors (38, 39), we hypothesized that the Ser polymorphism could impair the function of PHLPP2, thus providing a genetic mechanism driving constitutive phosphorylation of Akt in these cells. Comparison of

the Hs578t tumor cell line with its normal counterpart (Hs578Bst) revealed that the tumor cell line had a dramatic (>45-fold) increase in Ser 473 phosphorylation of Akt. Thus, the Ser allele tracks with increased Akt phosphorylation. Given that the Hs578t breast cancer cell line also expresses wild type PIK3CA (40), it provided further rationale that Ser1016 may be less functional and contribute to the increased phosphorylation of Akt in these cells.

Characterization of the L1016S substitution. To determine if Ser at codon 1016 alters phosphatase activity, we substituted a Ser for a Leu in the full length HA-PHLPP2 and expressed the Leu or Ser variants in H157 cells (possess only Leu Allele). We immunoprecipitated both variants and measured *in vitro* phosphatase activity towards fully phosphorylated Akt. We observed a decrease in phosphatase activity toward fully phosphorylated Akt at Ser 473 in the *in vitro* phosphatase assay when comparing it to the Leu1016, Fig. 2A. These data suggest that the amino acid change from a Leu to a Ser in the PP2C domain of the phosphatase decreases the activity of PHLPP2 towards Akt, and this could contribute to the increased phosphorylation of Akt in cells expressing the Ser variant compared to the Leu variant.

We next compared the effect of overexpressing the Ser1016 versus the Leu1016 variants on the phosphorylation state of Akt in cells. Expression of the Leu1016 construct in H157 cells resulted in a 5-fold reduction in phosphorylation of Akt at Ser473 compared to control cells (Figure 2B, lanes 1 and 2), consistent with previous results (19, 20). In contrast, expression of the Ser1016 construct at a

comparable level resulted in only a modest decrease in phosphorylation on Ser473 compared to control cells (Figure 2B, lane 3). To determine if the differential sensitivity of Akt to the two PHLPP variants would affect cellular processes PHLPP2 has been demonstrated to regulate, we expressed both variants in the H157 cells and monitored apoptosis. Figure 2C shows that expression of the Leu1016 variant increased apoptosis 5-fold; when expressed at comparable levels (see Figure 2B), the Ser1016 only increased apoptosis 2-fold. These results demonstrate that the Ser1016 variant has decreased activity towards Akt resulting in less effective suppression of the apoptotic phenotype.

Functional differences of endogenous PHLPP2 variants. To determine if endogenous PHLPP2 variants have altered activity, we compared the effect of knocking down PHLPP2 on Akt phosphorylation in the normal cell line Hs578Bst (expressing both alleles) to the pair-matched tumor cell line Hs578t (expressing only the Ser allele). Depletion of PHLPP2 (both variants) from the Hs578Bst cell line resulted in a robust increase in phosphorylation of Akt at Ser 473 (Figure 3A, lane 2) compared to control cells (Figure 3A, lane 1). In striking contrast, depletion of PHLPP2 (Ser1016 variant) from the tumor cell line, Hs578t, did not alter Akt phosphorylation. These data suggest that the Ser1016 variant is less functional compared to the Leu1016 variant and that only one functional allele is required for Akt regulation (note that Ser473 phosphorylation was not maximal in the Hs578t cells because addition of EGF caused an even greater increase in Ser 473 phosphorylation (data not shown)). Similar results were observed in the MCF-7 breast cancer cell line

(Figure 3B). Surprisingly, knock down of PHLPP1 from the Hs578t breast cancer cell line did not alter Akt phosphorylation, despite the lack of a mutation in PHLPP1 open reading frame. To confirm that the endogenous Ser1016 variant was less functional than the Leu1016 variant, we depleted PHLPP2 from two other breast cancer cell lines, MB-231 and ZR-75-1,that only express the Ser allele and monitored Akt phosphorylation. Depletion of the Ser1016 variant in these breast cancer cell lines did not alter Akt phosphorylation, confirming the impaired activity of this variant compared to the Leu variant (Figure 3B). Knock down of PHLPP1 from these breast cancer cell lines resulted in an increase in Akt phosphorylation (Figure 3B). Depletion of PHLPP2 from the breast cancer cell lines MCF-7 (which express both alleles) or Bt-474 (which only expresses the Leu allele) resulted in an increase in Akt phosphorylation, (Figure 3B). Similar results were observed for the SKBR-3 breast cancer cell line (19). These data reveal that the Ser1016 variant is ineffective towards suppressing the basal phosphorylation state of Akt.

To determine if the Ser1016 variant was also ineffective at regulating PKC, we depleted PHLPP2 in breast cancer cell lines expressing the Ser1016 variant, the Leu1016 variant, or both variants and monitored PKC protein levels. We observed an increase in PKCα protein levels following PHLPP2 depletion in breast cancer cell lines expressing the Leu1016 variant, (Figure 3C, lanes 10-15). In contrast depletion of PHLPP2 from cells expressing the Ser1016variant did not alter PKC protein levels (Figure 3A, lanes 3-8). The upregulation of PKC levels in cells expressing the Leu1016 variant correlated with the increased phosphorylation of Akt (e.g. compare

lanes 14 and 15 in Ser473 panel and PKCα panel). Interestingly, basal Akt phosphorylation was not detectable in MB-231 cell line despite these cells expressing only the less functional Ser1016 variant of PHLPP2; however, these cells had elevated PKCα protein expression, suggesting that the less functional PHLPP2 variant may specifically contribute to the increased expression of PKCα in this cell line.

To determine if the amino acid change from Leu to Ser at position 1016 altered the localization of endogenous PHLPP2, we fractionated breast cancer cells expressing either variant. Both the Leu1016 and Ser1016 variants were localized to the nucleus and cytoplasm (Figure 3D) suggesting that this amino acid change does not play a role in regulating the global cellular localization of PHLPP2.

We previously reported that the PHLPP family of phosphatases regulate both the amplitude and duration of agonist-induced Akt activation (19). Thus, we next asked whether the Ser1016 variant is less functional at regulating agonist-induced phosphorylation of Akt. We first compared the EGF-triggered phosphorylation of Akt in normal Hs578Bst cell line to that in its pair-matched tumor cell line, Hs578t. The Western blot in Figure 4A reveals a dramatic increase in Akt phosphorylation in Hs578Bst cells depleted of either PHLPP1 (lane 3) or PHLPP2 (lane 4) compared to vector-treated cells (lane 2) following treatment with EGF. In contrast, EGF-induced phosphorylation of Akt in Hs578t cells was not significantly altered following knockdown of PHLPP2 (Ser1016 variant) (lane 8). Nor did knockdown of PHLPP1 affect Akt phosphorylation. One possibility is that Akt is maximally phosphorylated so phosphatase depletion has no effect on Akt phosphorylation. To address this, we

measured the phosphorylation of Akt over a time course of EGF stimulation. The Western blot in Figure 4B reveals that depletion of PHLPP2 modestly increased the maximal phosphorylation of Akt (compare 5 min time points, lanes 2 and 6) and significantly prolonged the duration of phosphorylation (compare 60 min time points, lanes 4 and 8). We then tested the effect of PHLPP2 knockdown on agonist-induced activation of Akt in the remaining breast cancer cell lines expressing only the Ser1016 variant. The Western blots in Figure 4B reveal that depletion of PHLPP2 in the T47D, MB231, or Bt474 cells resulted in a dramatic increase in both the amplitude and duration of Akt phosphorylation. In fact, the magnitude of the knockdown effects were on the same order as that observed in the normal breast cell line Hs578Bst (Figure 4A). These data reveal that the Ser1016 variant is less functional at controlling Akt under basal or serum starvation conditions in all breast cancer cells, but effectively suppresses agonist-evoked phosphorylation of Akt.

Loss of the Leu allele from high-grade breast cancers. To determine if the Leu allele is lost in breast cancer tumor tissue compared to normal tissue from the same patient, similar to what we observed in the Hs578Bst-Hs578t paired cell lines, we genotyped pair-matched normal and tumor tissue samples from the same patient. Of the 33 patients we genotyped, eight possessed the polymorphism; of these eight, five were grade 2 ductal breast carcinomas and three were grade 3 ductal breast carcinomas (Figure 5A). Surprisingly, all three grade 3 breast cancer samples exhibited loss of the Leu allele (Figure 5A and 5B). We did not observe loss of either allele in the five grade 2 breast cancer samples (Figure 5A and 5C). These data suggest that

preferential loss of the Leu allele in high-grade breast cancers may contribute to the aggressive phenotype of these cancers by decreasing the basal phosphatase activity of PHLPP2, resulting in an increase in Akt phosphorylation, PKC protein levels, or both.

Examination of PHLPP2 variant allele frequencies in women discordant for breast cancer. To determine if women with the PHLPP2 polymorphism are predisposed to develop breast cancer, we genotyped 618 invasive breast cancers, 96 *in situ* breast cancers, and 583 population controls. Approximately 15% of controls carried a variant (Ser) allele. Carriers were at no greater risk of breast cancer when compared to noncarriers (age- and study site-adjusted odds ratio (OR): 1.01; 95% confidence interval (CI): 0.79-1.30; p=0.93). However, carriers of two variant alleles had a nonsignificantly reduced breast cancer risk (age- and site-adjusted OR: 0.56; 95% CI: 0.25, 1.26; p=0.16) (Table 1). This genotype was rare in the population (2.7% among controls).

Discussion

We have identified a nonsynonymous polymorphism in the PHLPP2 phosphatase that results in substitution of a Ser for a Leu at position 1016 in the PP2C domain. Biochemical analysis reveals that the less common variant, Ser1016, has impaired phosphatase activity towards the substrate Akt *in vitro*. Similarly, overexpression studies reveal that the Ser1016 variant is less effective at dephosphorylating Akt in cells, and thus less effective at inducing apoptosis compared to the Leu1016 variant. Consistent with impaired biological function, genetic

depletion of PHLPP2 in unstimulated (but not EGF-stimulated) cells expressing only the Ser1016 allele does not significantly affect either basal Akt phosphorylation or total PKC levels, whereas depletion of PHLPP2 in cells expressing the Leu1016 allele results in a robust increase in Akt phosphorylation and total PKC levels. We observe specific loss of the Leu1016 variant from a breast cancer cell line and high-grade breast cancer tumor tissue samples when compared to controls suggesting loss of the more functional variant may contribute to tumor progression. However, there is not an increased risk for the development of breast cancer in carriers of the Ser allele. These data highlight the complexity of the role of these PHLPP2 variants in breast cancer.

Identification of a nonsynonymous polymorphism in the PHLPP2 phosphatase. PHLPP2 is poised to be a potential tumor suppressor based on its chromosomal location and its regulation of downstream substrates, Akt and PKC, which are known oncogenes. The putative tumor suppressor genes on chromosome 16q remain elusive for ductal carcinomas. There is debate over the exact location of the SROs (smallest region of overlap) that contain the putative tumor suppressor genes in ductal carcinomas, but loss of 16q is a well-documented genetic event that occurs in both high-grade and low-grade ductal carcinomas (19). Additionally, a fragile site (FRA16B) is located within in the PHLPP2 gene, further implicating PHLPP2 as a potential tumor suppressor (NCBI Mitelman Breakpoint Map) (41). This prompted us to sequence breast cancer cell lines for somatic mutations, and led to the discovery of the L1016S polymorphism. In our initial studies we observed only the Ser allele in four breast cancer cell lines and confirmed that LOH is the mechanism responsible for

presence of only the Ser allele in one breast cancer cell line (Hs578t). This is likely the mechanism for the other three breast cancer cell lines as they have been previously described to have LOH at chromosome 16q (37). Additionally, we observed loss of the Leu allele in high-grade tumor tissue samples. This suggests that the Ser1016 variant plays a role in breast tumorigenesis, and prompted us to investigate the functional consequences of this amino acid change.

*Ser1016 variant is less functional than Leu1016 variant***.** Recent studies analyzing non-synonymous polymorphic variants demonstrate that polymorphisms can play a role in the development of cancer. The Stk15 T91A has been implicated to play a role in esophageal, renal and breast cancers and has been characterized as a low-penetrance tumor susceptibility gene (36, 42, 43). This underscores the need to continue to characterize nonsynonymous polymorphic variants and to determine if these variants play in a role in the generation or progression of cancer. Characterization of the endogenous Ser1016 variant demonstrated that it is less functional under basal conditions in all breast cancer cell lines examined. Surprisingly, this variant is still quite functional under agonist-induced conditions. We did observe that the Ser1016 is less functional under agonist-induced conditions in the Hs578t breast cancer cell line, but hypothesize that another mechanism may account for this loss of function since PHLPP1 is also less functional in these cells, despite lack of any somatic mutation. The overall observation that the Ser1016 is less functional under basal conditions suggests that loss of the more functional Leu allele will only increase Akt phosphorylation and PKC protein levels modestly. This modest

increase in activity and protein levels could contribute to the overall proliferative and metastatic potential of breast cancer cells and could explain why we observe loss of the more functional allele in breast cancer cell lines as well as tumor tissue samples. This could contribute to increased cell proliferation by increasing activity of Akt3, which has been demonstrated to play a role in breast cancer (29, 30), leading to increased phosphorylation of p27. We have previously defined this as a unique axis of signaling involving PHLPP2, Akt3, and p27 (19, 31). Depletion of PHLPP2 results in increased p27 phosphorylation, and this increase can be rescued by depletion of Akt3 in normal breast cells (19, 31). Therefore, it is not unreasonable to hypothesize that loss of a more functional PHLPP2 may increase Akt3 activity, resulting in increased p27 phosphorylation, causing cytosolic sequestration of p27 and increased proliferation. Interestingly, p27 has been demonstrated to be localized to the cytoplasm as a result of Akt phosphorylation in women with breast cancer and this correlates with poor survival rates (44, 45).

Loss of specific regions of chromosome 16q has been associated with lower grade, non-metastatic breast cancers that do not recur, but these regions do not include the PHLPP2 gene (33). Interestingly, a region that includes the PHLPP2 gene was identified to play a role in metastatic breast cancer (46). These data suggest that PHLPP2 may not be involved in the early stages of tumorigenesis, but rather play a role in metastasis. This is consistent with the observation of decreased survival rates in women with p27 localized to the cytoplasm as a result of Akt phosphorylation (44, 45).

The observation that PHLPP2 remains functional under agonist-induced conditions in all cancer cell lines examined, except the Hs578t breast cancer cell line, suggests PHLPP2 could make an excellent therapeutic target. Similar results were observed for PHLPP1. If small molecule compounds can be discovered that result in activation or increased expression of these phosphatases this could result in loss of activation of both PKC and Akt, and ultimately lead to tumor regression.

Are Ser/Ser homozygotes at decreased breast cancer risk? We observed fewer Ser/Ser homozygotes among cases than controls in the case-control study population, suggesting a possible protective influence of this genotype in breast cancer. This was an unexpected result, and chance is the most likely explanation (the result was not statistically significant). However, we note that the variant lies approximately 19kb up/ downstream from a SNP (rs11075895) in PHLPP2 (also known as KIAA0931) found to have a highly significant inverse association with breast cancer risk in a genome wide association study ((http://caintegrator.nci.nih.gov/cgems/). Assuming these variants are in linkage disequilibrium, the results are compatible with a functionally relevant polymorphism (this one or another) somewhere in this region of the gene. If not due to chance, one possible explanation for the current inverse association we observed is that women who are homozygous for the Ser allele never have the opportunity to lose a more functional allele. Thus, cells may have activated compensating mechanisms to account for the presence of the less functional PHLPP2 phosphatase from development. Alternatively, these women may have modestly increased levels of Akt1 kinase activity because of the less functional PHLPP2 variant,

and this could contribute to the lower rates of breast cancer observed in these women. Additionally, the Ser allele could contribute to the development of other diseases in which Akt function is perturbed, notably type II diabetes, heart disease, or other forms of cancer (47). The Ser/Ser genotype appears to be quite rare in Caucasians: only 16 of 583 control women in the case-control study (2.7%) and no person in the 100 Coriell samples we examined (all Caucasian) harbored this genotype.

Summary. Our data identify a novel polymorphic variant of the PHLPP2 phosphatase that occurs in 15% of the population. This polymorphic variant, Ser1016, has impaired phosphatase activity towards Akt and is less effective at suppressing the basal phosphorylation of Akt compared to the common variant, Leu1016. The less functional allele is preferentially retained in a breast cancer cell lines as well as high-grade breast tumor tissue samples. Finally, we observed a decreased frequency of breast cancer in women homozygous for the Ser allele, though this unexpected result was not statistically significant and could well have emerged by chance. Further epidemiologic studies are needed to fully explore the associations of this and other variants in PHLPP2 as potential determinants of human breast cancer risk.

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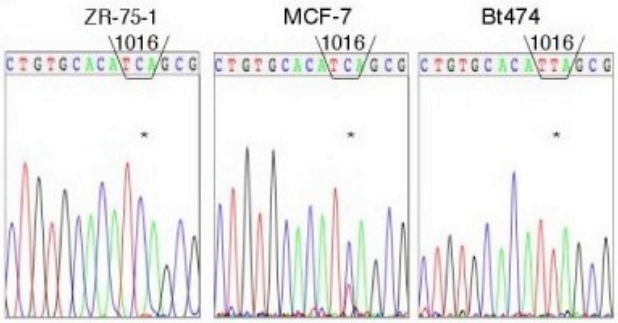
A



B

Breast Cancer Cell Line	Codon 1016 Amino Acid
MDA-MB-436	Leu
ZR-75-30	Leu
MDA-MB-134	Leu
CAMA-1	Leu
MDA-MB-435	Leu
ZR-75-1	Ser
MDA-MB-231	Ser
MCF-7	Ser/Leu
Hs578t	Ser
T47D	Ser
SKBR3	Leu
MDA-MB-468	Leu
Bt474	Leu
HCC1187	Leu
HCC1143	Leu
HCC1395	Leu
HCC1945	Leu
HCC38	Leu

C

Patient Status	Homozygous Leu allele	Heterozygous	Homozygous Ser allele
Breast Cancer	25	8	0
Heathy Women	37	12	0
Healthy Men	33	18	0

D

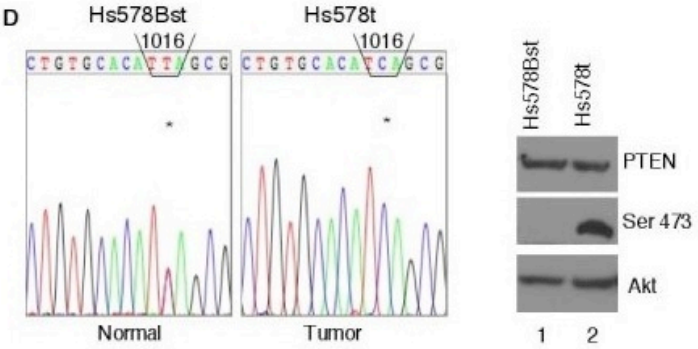


Figure 3.1. Identification of the L1016S Polymorphism in the PHLPP2 Phosphatase. *A,* Chromatograms from the indicated breast cancer cell lines that possess only the Ser allele, both alleles, or only the Leu allele. RT-PCR was performed using primers flanking the polymorphism and PCR products were then sequenced using nested primers. The nucleotide (C or T) at position 3047 is indicated with an asterisk. *B,* Table displaying genotype of 18 breast cancer cell lines. *C,* Genotype of 100 healthy individuals from the Corriel Institute and 33 pair match breast cancer patients, confirming nucleotide change is indeed a polymorphism. *D,* Comparison of the normal cell line Hs578Bst and the tumor cell line Hs578t from the same patient showing presence of T and C at position 3047 in normal cell line but only C in tumor cell line (asterisk). Western blot of lysates from Hs578Bst (lane 1) or Hs578t (lane 2) cells probed with antibodies for PTEN, Akt, or Akt phosphorylated at Ser 473.

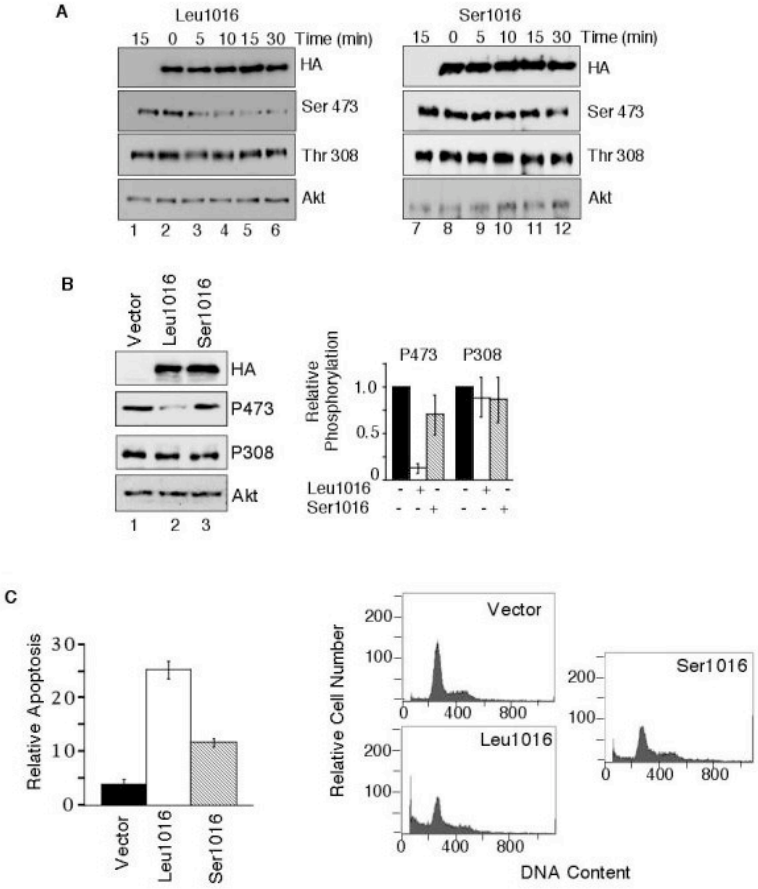


Figure 3.2. Characterization of amino acid change in the PHLPP2 phosphatase. *A,* H157 cells were transfected with vector (lanes 1 and 7), HA-Leu1016 (lanes 2-6), or HA-Ser1016 (lane 8-12) under high serum conditions (10% FBS DMEM) for 48 hr.; thereafter HA-PHLPP2 variants were immunoprecipitated and incubated with pure phosphorylated Akt for the indicated times. Akt protein and phosphorylation was detected using Akt and phospho-specific antibodies, respectively. Western blots are representative of three independent experiments. *B,* H157 cells were transfected with vector (lane 1), HA-Leu1016 (lane 2), or HA Ser1016 (lane 3) for 48 hr. under high serum conditions prior to lysis. The phosphorylation state of Akt in lysates was detected by Western blot analysis. Data from three independent experiments are summarized in the bar graph (relative phosphorylation of Akt at P473 or P308 was normalized to total Akt). Error bars indicate standard deviation. *C,* H157 NSCLC cells were transfected with vector, HA-Leu1016, or Ser1016, under low serum conditions (0.1% FBS DMEM) for 48 hr. and apoptosis (sub-2N DNA content) was assessed using propidium iodide incorporation assays and flow cytometry. All assays were performed in triplicate, with error bars indicating standard deviation, and are representative of three independent experiments.

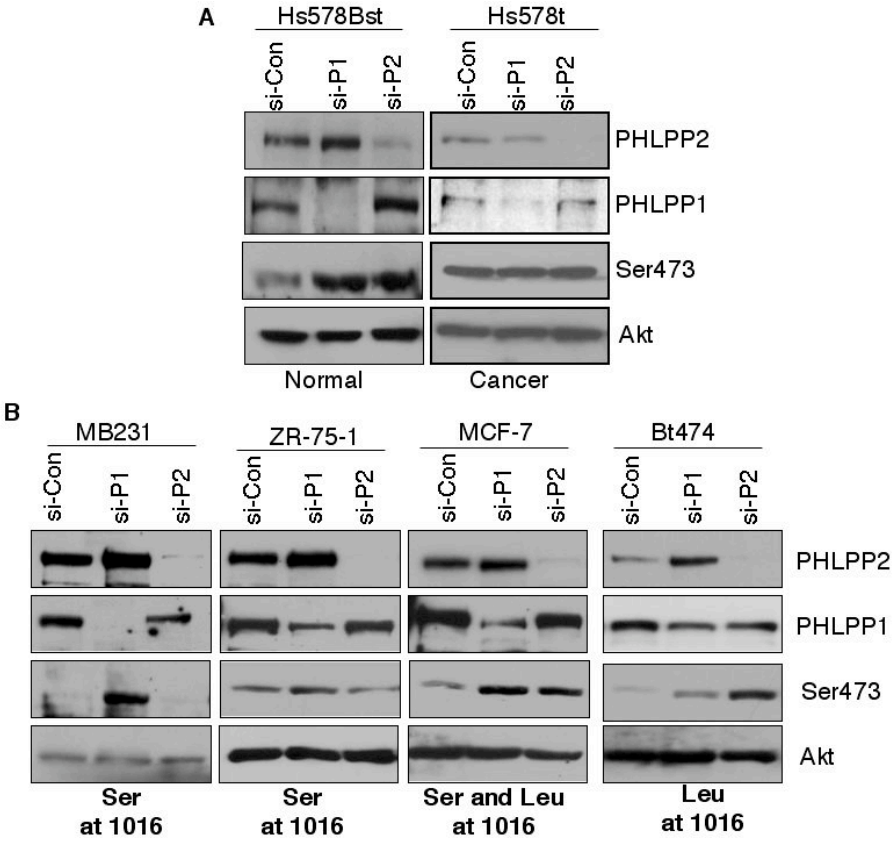


Figure 3.3. Evaluating functionality of endogenous Ser1016. *A and B,* Hs578Bst, Hs578t, MB231, ZR-75-1, MCF-7 and Bt474 cells were transfected with non-targeting siRNA control (si-Con), PHLPP1 smartpool siRNA (si-P1), or PHLPP2 smartpool siRNA (si-P2), for 48 hrs. under high serum conditions and lysates were analyzed by Western blot. The phosphorylation state of Akt at Ser 473, and relative protein levels of Akt, PHLPP1, and PHLPP2 were detected using the indicated antibodies. Western blots are representative of three independent experiments.

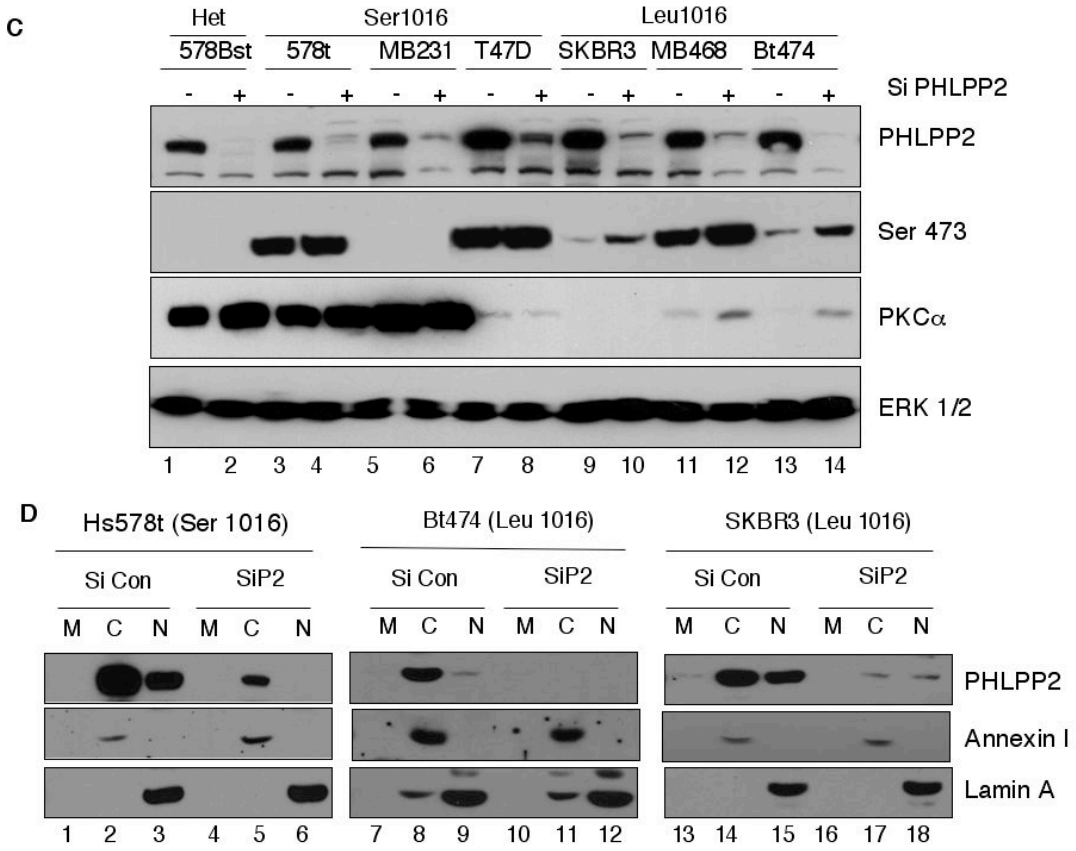


Figure 3.3: *C,* The indicated breast cancer cells were transfected with non-targeting siRNA control (si-Con) or smartpool siRNA to PHLPP2, incubated 48 hr. under high serum conditions and then lysates were analyzed by Western blot. The phosphorylation state of Akt at Ser 473, and relative protein levels of Akt, PKCα, ERK1/2, and PHLPP2 were detected using the indicated antibodies. Western blots are representative of three independent experiments. *D*, Fractionation of endogenous PHLPP2 variants was performed and membrane, cytoplasmic and nuclear fractions analyzed by Westesrn blot. Protein levels of PHLPP2, annexin I, and lamin A were detected using indicated antibodies. Western blots are representative of three independent experiments.

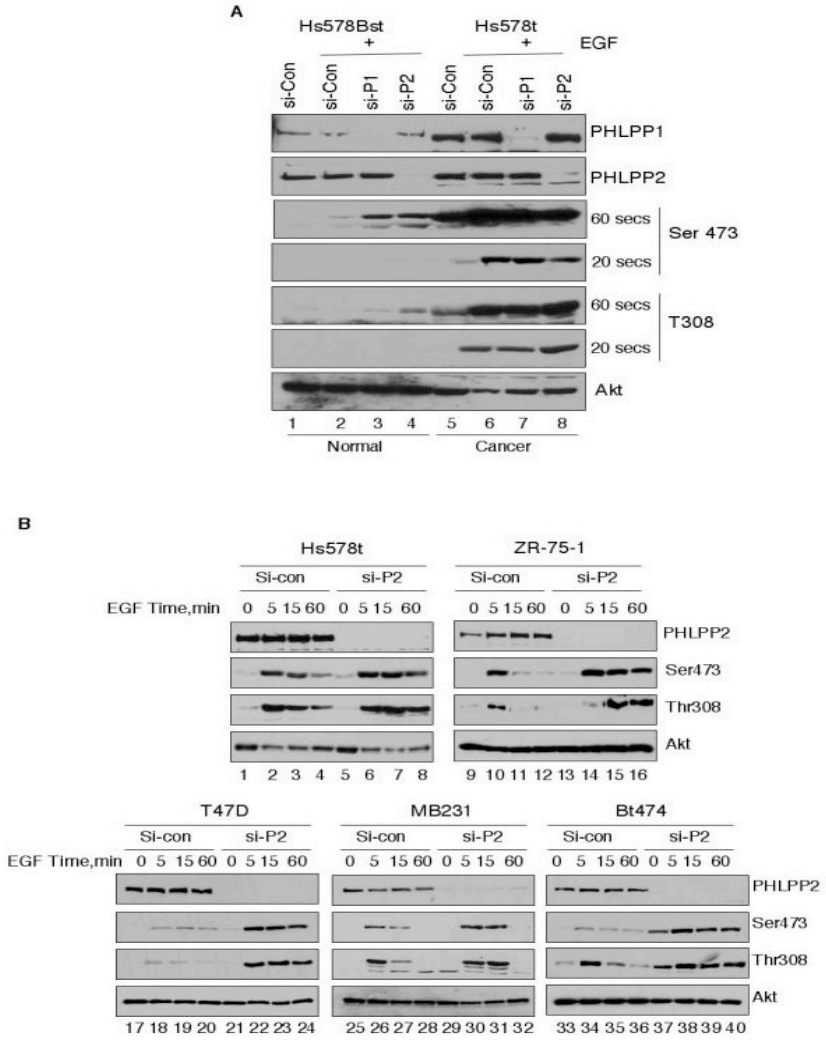


Figure 3.4. Agonist-induced regulation of Akt by PHLPP2 variants. *A*, Hs578Bst and Hs578t cells were transfected with non-targeting siRNA control (si-Con) or smartpool siRNA to PHLPP1 or PHLPP2 under high serum conditions and incubated for 48 hr. Media was then changed to low serum conditions overnight prior to addition of EGF (10 ng/ml) for 15 min. The phosphorylation state of Akt at Ser473 and Thr308, and protein levels of Akt, PHLPP1 and PHLPP2 in lysates were detected by Western blot analysis. Western blots are representative of three independent experiments. *B*, Indicated breast cancer cell lines were transfected with non-targeting siRNA control (si-Con) or smartpool siRNA to PHLPP2 under high serum conditions and incubated for 48 hrs. Media was then changed to low serum conditions overnight prior to addition of EGF (10 ng/ml) for the indicated time. The phosphorylation state of Akt at Ser473 and Thr308, and protein levels of Akt and PHLPP2, in lysates were detected by Western blot analysis. Western blots are representative of three independent experiments.

A

Breast Cancers with Polymorpism	Total	Loss of Ser allele	Loss of Leu Allele	Loss of No Allele
Grade 2 Breast Cancers	5	0	0	5
Grade 3 Breast Cancers	3	0	3	0

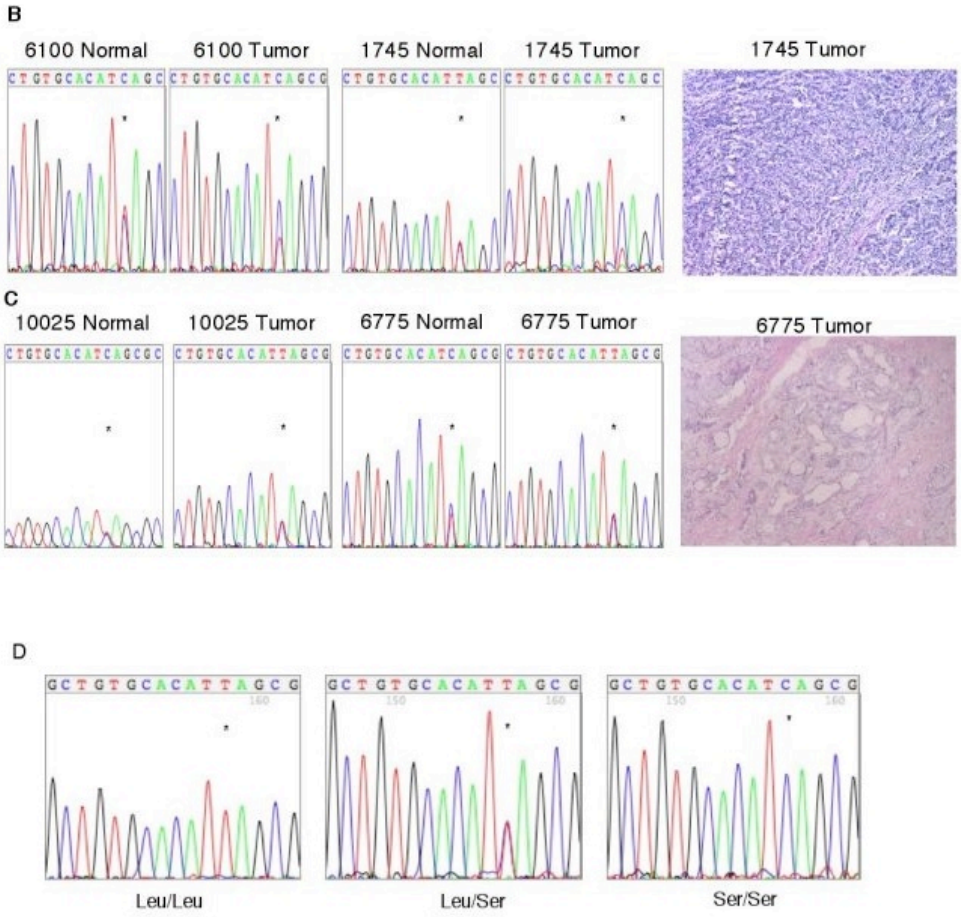


Figure 3.5. Assessment of PHLPP2 variants in normal and tumor tissue from the same patient. *A,* Table indicating genotypes of breast tumors that were heterozygous for both PHLPP2 alleles. All high-grade breast cancer samples displayed LOH of the Leu1016 variant of PHLPP2. *B,* Chromatograms of two high-grade tumors and pair-matched control tissue showing presence of T and C at position 3047 in control tissue but only C at position 3047 in tumor tissue, resulting from loss of the Leu allele in breast cancer samples. Histology displaying representative high-grade breast tumor samples. C, Representative chromatograms of two low-grade breast tumors showing presence of both C and T at position 3047 resulting from retention of both PHLPP2 alleles. Histology of a representative low-grade breast cancer. D, Representative chromatograms from US population-based case-control study indicating women homozygous for the Leu allele, heterozygous, or homozygous for the Ser allele.

Table 3.1. Risk of breast cancer according to the L1016S PHLPP2 genotype in a US population-based case-control study

	Invasive Breast Cancer			In Situ Breast Cancer			All Breast Cancers		
Genotype	Case (618)	Ctrl (559)	OR (95% CI)	Case (96)	Ctrl (583)	OR (95% CI)	Case (714)	Ctrl (583)	OR (95% CI)
LEU/LEU	449	408	1.00 (ref)	67	425	1.00 (ref)	516	425	1.00 (ref)
LEU/SER	159	136	1.04 (0.79,1.36)	29	142	1.08 (0.65,1.78)	188	142	1.06 (0.82,1.37)
SER/SER	10	15	0.67 (0.29,1.53)	0	16	na	10	16	0.56 (0.25,1.26)

Risk of breast cancer according to the L1016S PHLPP2 genotype in a US population-based case-control study. OR = odds ratio; CI = 95% confidence interval. ORs and CIs adjusted for age and study center. Control series includes women up to age 69 years for invasive breast cancer, and up to age 74 years for *in situ* breast cancer. The test for gene dose effect under an additive model was nonsignificant for invasive breast cancer (p= 0.79), and invasive and *in situ* breast cancer combined (p=0.74).

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Chapter 4

The Three Ps of PHLPP: PH domain, PKC and the PDZ binding motif

Abstract

Many questions still remain regarding the phosphatase PHLPP. The aim of this chapter is to address a subset of these questions. Specifically, this chapter will focus on research aimed at determining if the other signaling domains of PHLPP are functional and if they participate in regulating signaling pathways, assessment of other downstream targets of PHLPP, and lastly determining if PHLPP is somatically mutated in cancer? Functional analysis of the PH domains of the PHLPP phosphatases suggests these domains can bind phosphoinositides with a similar profile as the Akt PH domain. Furthermore, we have identified a missense somatic mutation in the PH domain of the PHLPP2 phosphatase. Additionally, we have identified a novel PHLPP2 interacting protein; scribble that is a putative tumor suppressor. These data suggest that these domains are functional and that the PH domain could be the target of somatic mutations in breast cancer. Finally, we will examine the mechanism by which PHLPP regulates PKC activity: by dephosphorylating the hydrophobic motif of PKC, PHLPP promotes the degradation of PKC. These data will hopefully provide directions for future avenues of research.

Introduction

Protein signaling domains play an imperative role in dictating the function of signaling molecules such as kinases and phosphatases (1). It is important to dissect the function of these signaling domains in order to better understand how these molecules dictate cellular processes. Similarly, it is important to understand all substrates for a given phosphatase or kinase in order to understand the array of biological functions given signaling molecules regulate within a cell. To this end we have attempted to further understand the role of the PH domain and PDZ binding motif in regulating the function of the PHLPP phosphatases. Data demonstrate that the PDZ binding motif of PHLPP is critical for its regulation of Akt, while the PH domain is important for its regulation of PKC (2, 3). To better understand these observations we examine if the PHLPP phosphatases have functional PH domains and aim to identify novel interacting proteins that contain PDZ domains. Furthermore, we have set out to determine if the PH domain plays a role in cancer by searching for somatic mutations in this domain. There is precedence for PH domain mutations playing a role in cancer (4).

Lastly, identification of novel substrates of the PHLPP phosphatases should help us to understand how these phosphatases regulate proliferation and cellular survival (3, 5). We have identified the novel substrate PKC, which is a member of the AGC family of kinases. We observe that PHLPP dephosphorylates PKC directly at the hydrophobic motif, and this leads to decreased stability and degradation of members of PKC family of kinases. Endogenous PHLPPs play important role in

setting the level of PKC kinases expressed in cells as depletion of these phosphatases results in an increase in PKC expression.

Methods

Materials. Smartpool siRNA against PHLPP1 and PHLPP2 was purchased from Dharmacon. PHLPP1 and PHLPP2 isoform-specific antibodies were purchased from Bethyl Laboratories. GST, PKCα, PKCβII, PKCε, and Scribble antibodies were purchased from Santa Cruz Biotechnology. HA (12CA5) antibody was purchased from Roche. PIP strips were purchased from Echelon. Breast tumor tissue was provided by Dr. Linda Wasserman. The Akt GST-PH domain construct was provided by Dr. Alex Toker.

PH domain Proteins. A GST-tagged construct of the PH domain for bacterial expression was generated by amplifying the coding region of the PH domain (corresponding to residues 23-125 PHLPP1α and 150-240 PHLPP2) by PCR and sub-cloning the sequence into *EcoR I* and *Xho I* sites of pGEX-KG vector.

PIP strip Assays. Assays were performed following manufacturer's protocol (Echelon). Briefly, 0.5μg/μl PH domain protein or 300μl of HA-PHLPP1α transfected cell lysates were incubated overnight at 4°C with a single PIP strip. For detection of bound protein, membranes were incubated with anti-GST monoclonal antibody or anti-HA monoclonal antibody.

Cell transfections and immunoblotting. All cell lines were maintained in DMEM (Cellgro) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained at 37°C in 5% CO_2. Transient transfections and siRNA experiments

were performed as previously described. Transfection efficiencies for 293T cells averaged between 70-90% for each experiment. For immunoblotting, transfected cells were lysed in Buffer A (50 mM Na_2HPO_4, pH 7.5, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% SDS, 1 mM DTT, 200 mM benzamidine, 40 mg ml^{-1} leupeptin, and 1 mM PMSF) and sonicated for 5 seconds. Lysates containing equal protein were analyzed on SDS-PAGE gels, and individual blots were probed using indicated antibodies.

In vitro phosphatase assay. A GST-tagged fusion protein of PHLPP1-PP2C was expressed and purified as described previously (2). His-tagged PKCβII was expressed and purified from baculovirus-infected Sf21 cells. Briefly, Sf21 cells were maintained in SF-900 II media (Invitrogen) and infected with baculovirus encoding His-PKCβII for 3 days. The infected cells were lysed in PBS containing 1% Triton X-100 and 10 mM imidazole, and His-PKCβII proteins were purified using Ni-NTA beads (Qiagen). The dephosphorylation reactions were carried out in a reaction buffer containing 50 mM Tris (pH 7.4), 1 mM DTT and 5 mM $MnCl_2$ at 30°C for 30 minutes. The final concentration of His-PKCβII and GST-PP2C in the reactions were 50 nM and 10 nM, respectively.

Immunoprecipitation. Immunoprecipitation of HA-PHLPP2 was performed as described previously. Briefly, the transfected cells were lysed in Buffer B (50 mM Na_2HPO_4, pH 7.5, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% triton, 1 mM DTT, 200 mM benzamidine, 40 mg ml^{-1} leupeptin, and 1 mM PMSF), sonicated for 5 seconds and precleared, and the detergent-solubilized cell

lysates were subjected to immunoprecipitation using the anti-HA monoclonal antibody to precipitate HA-PHLPP2. The immunoprecipitates were washed three times in Buffer C 2 (20 mM HEPES, pH 7.4, 1% Triton X-100, 1 mM DTT, 200 mM benzamidine, 40 mg ml^{-1} leupeptin, and 1 mM PMSF). Bound proteins were analyzed by SDS-PAGE and immunoblotting.

DNA Isolation. Genomic DNA was isolated from human tissues using QIAamp DNA mini kit (Qiagen) following the manufacturer's protocol. Tissue samples were incubated in ATL buffer (from kit) supplemented with proteinase K for 3 hrs at 56° C prior to DNA extraction.

Genotyping. 10ng of DNA was added to PCR sequencing buffer (5µl reaction mix (Stratagene), 200nM of each primer, 1µl Pfu tubro DNA polymerase (stratagene), 40.5µl water (50µl total reaction amount)). The following PCR scheme was used for each reaction: 2min. at 95 ºC, (1 min. at 94 ºC, 1 min. at 55 ºC, 1 min at 68 ºC) x 35, 10 min. at 68ºC, 4 ºC thereafter. The DNA-PCR products were sequenced directly using nested PCR primers. The DNA primers used for PCR and sequencing were 5'–ggctgttggaagaagggtta–3' and 5'- ttggggcataatagcctctg –3'. Nested primers are as follows: 5'-atgtaacaatggcatccaaagtac-3' and 5'- gcccatactggcttctttga-3'.

Results

In an effort to understand if the PH domain of PHLPP is functional we incubated purified bacterially expressed PH domain from both PHLPP1 and PHLPP2 with a membrane containing various phosphorylated phosphatidyl inositides (PIP

strip). As a control we used the purified bacterially expressed PH domain of Akt. We observed that PHLPP1, PHLPP2 and Akt PH domains bind to PI(4)P, PI(5)P, and PI(3,5)P_2 (Figure 1A). In addition the PH domain of Akt and PHLPP2 bind to PI(3,4,5)P_3 (Figure 1A). To verify that full-length PHLPP1 had similar specificity for phosphorylated phosphoinositides we incubated a PIP strip with cell lysates from cells transfected with HA-PHLPP1 from 293T cells and detected bound HA-PHLPP1 with anti-HA antibodies. We observed that full-length PHLPP1 preferentially bound to PI, PI(3)P and PI(3,5)P_2 (Figure 1B) suggesting there may be some conformational change present in full-length PHLPP1α that alters the specificity of the PHLPP1 PH domain. These data suggest that both PHLPP1 and PHLPP2 possess functional PH domains with varying degrees of preference for phosphorylated phosphoinositides.

The observation that both PHLPP1 and PHLPP2 appear to have functional PH domains suggest that these domains could be mutationally targeted in cancer to limit targeting of the PHLPPs to various intracellular membranes. This could result in loss of regulation of downstream targets such as PKC (2). In an effort to determine if the PH domain is somatically mutated, we sequenced the PH domain of PHLPP2 in both normal and tumor tissue from the same patient. We observed one missense somatic point mutation in exon 3, codon 190, that results in an A->C nucleotide change at position 570 in the open reading frame. This results in an amino acid change of Q in normal tissue to H in tumor tissue (referred to as Q190H). This mutation has been identified in three different patient tumor samples out of eleven (27%). One of these samples retains a copy of the wild-type allele and a copy of the mutated allele (Figure

2A). Surprisingly, two of these samples only possess the mutated allele, suggesting that the somatic mutation occurred followed by loss of the wild-type allele (figure 2B). Further studies need to be performed to determine if this point mutation results in loss of function of the PHLPP2 gene.

In an effort to identify proteins that interact with PHLPP phosphatases, we hypothesized that proteins with leucine rich repeats and PDZ domains could interact with PHLPP. Recently a new family of proteins has been characterized, referred to as LAP proteins, for leucine rich repeat and PDZ domain, which act as scaffolding proteins (6). We examined the interaction of PHLPP2 with LAP family members scribble and erbin. Our data suggest that scribble does associate with PHLPP2, as determined through co-immunoprecipitation experiments (Figure 3). The next step would be to determine if scribble plays a role in regulating PHLPP2-meidated dephosphorylation of Akt (3).

There are approximately 400 Ser/Thr kinases in the human genome yet only 30 Ser/Thr phosphatases (7), suggesting that phosphatases will be promiscuous and regulate multiple downstream substrates. To identify novel substrates of PHLPP phosphatases we focused on Akt's close cousin, PKC. Both Akt and PKC have the conserved hydrophobic motif, which is required for full catalytic activation of the kinase (8). To determine if PHLPP could regulate phosphorylation at the hydrophobic motif of PKC, we incubated the purified bacterially expressed PP2C domain of PHLPP1 with phosphorylated PKCβII in vitro. The PKCβII is purified from insect cells and is phosphorylated at all three conserved sites including Thr 500, Thr 641, and

Thr 660 (2). Incubation with the PHLPP1 PP2C domain revealed that it dephosphorylates Thr 641 (turn motif) and Thr 660, but not Thr 500, at the activation loop. These data reveal that the PHLPP phosphatase domain can directly dephosphorylate both the hydrophobic motif and turn motif of PKC in vitro (Figure 4A).

To ascertain if PHLPP was regulating PKC in cells, PKCβII was co-expressed with HA-PHLPP1 or an empty vector in the 293T cells and distribution of PKC was monitored. When PKC is dephosphorylated it is shunted to the insoluble pellet and targeted for degradation (9). In empty vector transfected control cells PKCβII migrates as a fully phosphorylated mature species in the soluble fraction of the cells (Figure 4B). Co-expression of PHLPP1α results in a faster migrating species of PKC, consistent with PHLPP causing dephosphorylation at this site (arrow, single asterisk, Figure 4B). This slower migrating species of PKC accumulates in the detergent insoluble fraction of cells. These results reveal that co-expression of PHLPP1α with PKCβII in cells promotes the dephosphorylation and accumulation of dephosphorylated PKC in the detergent insoluble fraction of cells, where it can be targeted for degradation.

To determine if endogenous PHLPP phosphatases were controlling the phosphorylation, and thus the protein levels of PKC in cells, endogenous PHLPP1 and PHLPP2 were depleted from normal breast cells and PKC protein levels were monitored. Depletion of endogenous PHLPP1 and PHLPP2 results in increased protein expression of PKCα, PKCβII and PKCε (Figure 4C). These data are

consistent with PHLPP phosphatases regulating the phosphorylation of PKC to control the protein levels of PKC in cells. Modulation of PKC protein levels acutely regulates the signaling of the PKC pathway, thus the elucidation that PHLPPs control signaling via the PKC pathway.

Discussion

To fully understand the role of protein-protein interaction domains in guiding protein function it is important to determine if these domains are active, or just pseudo-domains that are nonfunctional (1). We have examined the functionality of the PH domain in vitro and observed that this domain is functional towards specific phosphoinositides. Interestingly, the PH domain of PHLPP1 and PHLPP2 appear to have a similar PIP binding profile to the PH domain of Akt. A similar profile was not observed for the PDK-1 PH domain, suggesting these are specific interactions (data not shown). However, binding of the full-length PHLPP1α was not identical to the PH domain of PHLPP1α alone. This interaction should be re-examined using pure preparations of PHLPP1α and PHLPP2. Further studies need to be performed to ascertain if the PHLPPs bind phosphoinositides in vivo.

Given that the PH domain is functional it is important to determine if this domain is targeted during the process of tumorigenesis. PH domains can be auto-inhibitory and mutations that alter this function can result in constitutive activation of a kinase, such as Akt (10), or possibly constitutive inhibtion of a phosphatase such as PHLPP2. Alternatively, a missense point mutation in the PH domain could result in mislocalization of the protein due to altered specificity for phosphoinositides and

result in loss of regulation of downstream substrates (4). Sequencing of exon 3 of the PHLPP2 gene revealed a novel somatic mutation that results in an A->C nucleotide change at position 570 in the open reading frame. This results in an amino acid change from Q to H at codon 190 in the PH domain. The functional role of this point mutation should be further examined to determine if it alters PHLPP2 phosphatase activity in vitro and in vivo. Future studies should explore the role of this point mutation in the process of tumorigenesis.

Identification of novel protein interacting partners can provide insights into cellular localization of proteins and biological function (1). Often novel interacting proteins are scaffolding proteins that dictate targets of enzymes (1). We have identified a member of the LAP family of proteins, scribble, as a novel interactor of PHLPP2. This protein family includes the novel tumor suppressor scribble and ERBIN, which binds to the ERBB2 receptors (11). Scribble plays a role in regulating both cell proliferation and polarity, and flies that lack scribble undergo hyper-proliferation and experience loss of apical-basal polarity (12). Interestingly, erbin did not bind to PHLPP2. PDZ bioinformatic motif analysis is consistent with our result that scribble is a novel interacting protein for PHLPP2 (13). This interaction should be examined further, since it is possible that scribble localizes PHLPP2 to proliferation specific substrates.

Lastly in an effort to identify novel substrates of the PHLPP family of phosphatases we examined PHLPPs ability to regulate another AGC kinase family member, PKC. The phosphorylation state of PKC controls its stability, as best

illustrated by the finding that the enzyme is depleted in cells lacking the upstream kinase, PDK-1 (14). Because the phosphorylation of PKC is constitutive, dephosphorylation mechanisms are poised to play a key role in controlling the amount of PKC and thus the amplitude of the PKC signal. Here, we identified PHLPP1 and PHLPP2 as regulators of PKC phosphorylation in cells. We showed that both phosphatases dephosphorylate PKCβII on the hydrophobic motif, an event that shunts PKCβII to the detergent-insoluble fraction of cells. In addition, depletion of endogenous PHLPP1 or PHLPP2 via siRNA in normal breast epithelial cells results in a marked increase of endogenous PKC expression. Thus, PHLPP phosphatases control the level of cellular PKC by dephosphorylating a key site, the hydrophobic motif, which controls the stability of this family of kinases.

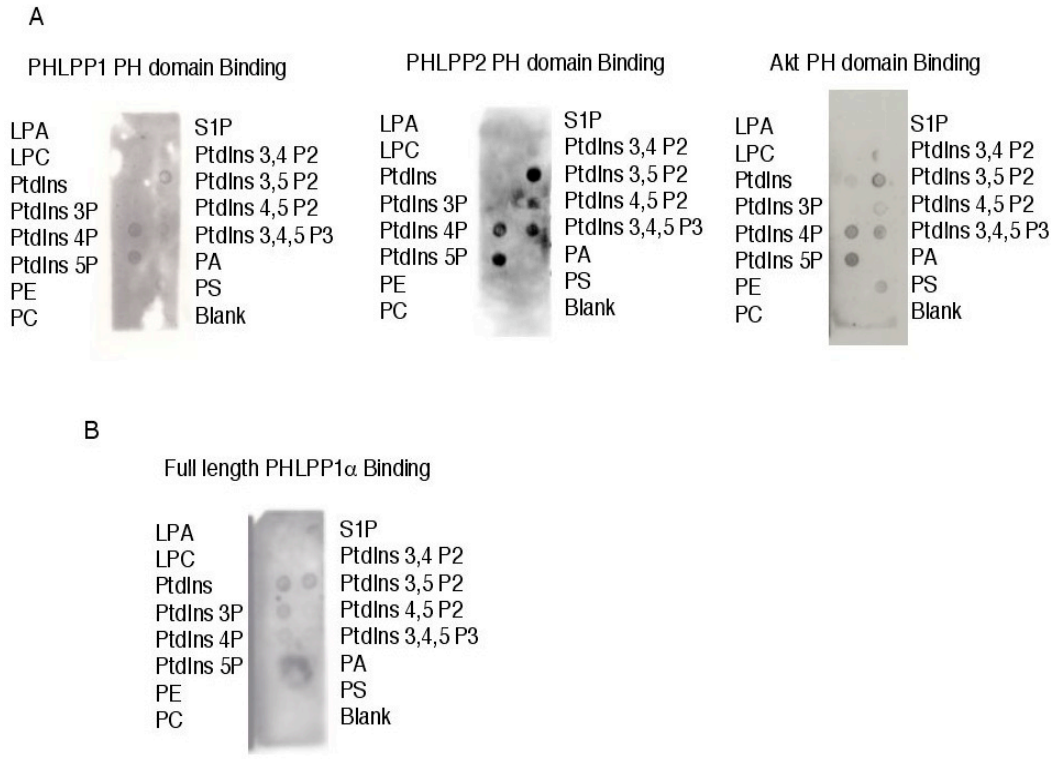

Figure 4.1: PHLPP PH domain binds various phosphoinositides in vitro. (A) The PIP strip reactions were carried out by incubating GST-PH domain of PHLPP1, PHLPP2 or Akt. Protein binding was detected using an anti-GST monoclonal antibody. (B) HA-PHLPP1α was transfected into 293T cells and cell lysates were incubated with a PIP strip. Bound PHLPP1α was detected using anti-HA monoclonal antibody.

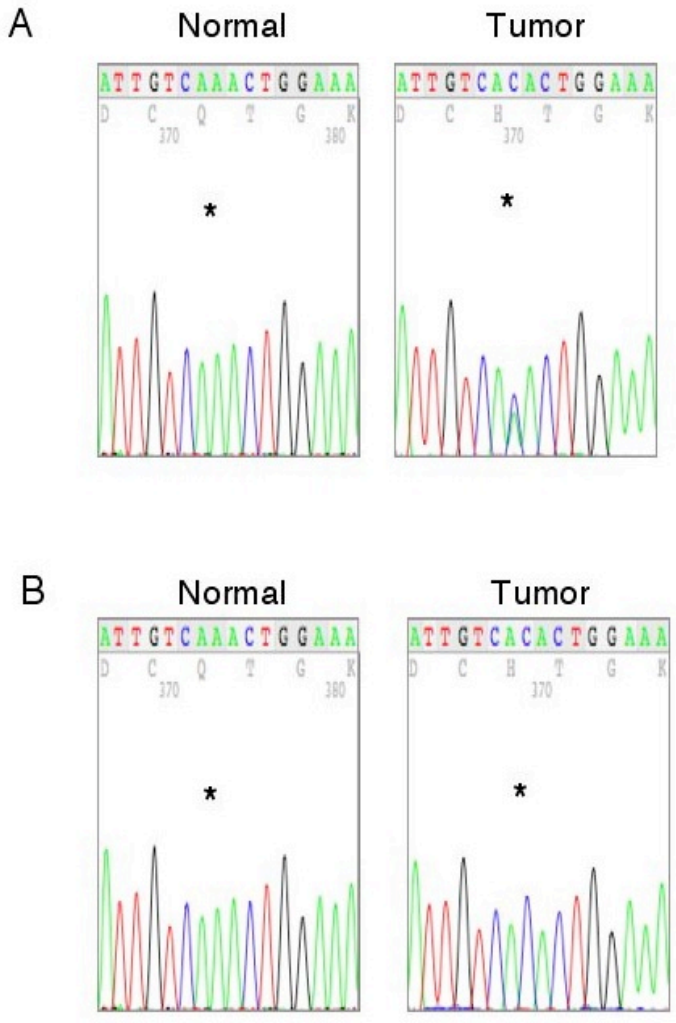

Figure 4.2: PHLPP2 is somatically mutated in breast cancer. Chromatograms displaying A->C somatic mutation that results in a Q190H amino acid change in the PH domain.

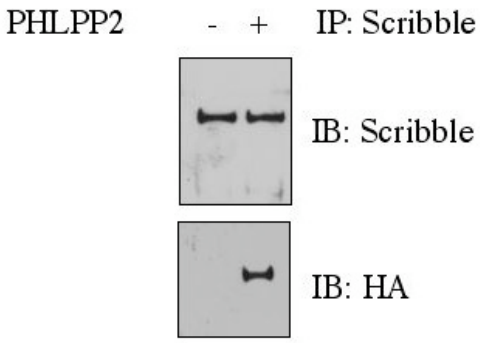

Figure 4.3: Co-immunoprecipitation of PHLPP2 and scribble. 293T cells were transfected with HA-PHLPP2 and the detergent-soluble supernatants were immunoprecipitated with the anti-HA monoclonal antibody. Immunoprecipitated proteins were analyzed using SDS-PAGE and immunoblotting. The presence of scribble in the immune complexes was detected using the scribble antibody, and the amount of HA-tagged proteins in the immunoprecipitates was detected by the anti-HA (12CA5) monoclonal antibody.

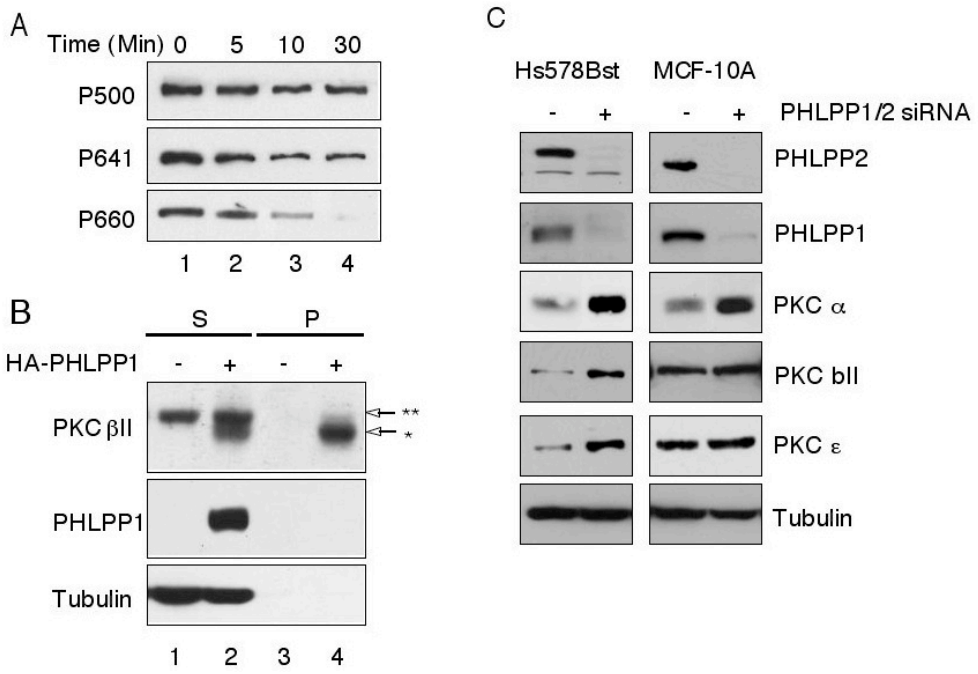

Figure 4.4: PHLPP1 dephosphorylates PKC *in vitro* and *in vivo*. (A) Dephosphorylation of PKCβII by purified PP2C domain of PHLPP1. The dephosphorylation reactions were carried out by incubating purified His-PKCβII with purified GST-PP2C for 0-30 min (lanes 1-4). The dephosphorylation of PKCβII I at T500, T641 or S660 was detected by the phospho-specific antibodies P500, P641 or P660, respectively. (B) Dephosphorylation of PKCβII upon overexpression of HA-PHLPP1 in cells. COS7 cells were transfected with PKCβII together with either vector (lanes 1 and 3) or HA-PHLPP1 (lanes 2 and 4). The cells were fractionated into detergent-soluble supernatants (S, lanes 1 and 2) and detergent insoluble pellets (P, lanes 3 and 4). The appearance of faster-migrating species of PKCβII indicates dephosphorylation of the protein (labeled by a dash (*)), while the mature fully-phosphorylated species is labeled with two asterisks (**). The expression of PKCβII and HA-PHLPP1 was detected by the anti- PKCβII antibody (upper panel) and the anti-HA monoclonal antibody (middle panel), respectively. Tubulin was detected with an anti-tubulin monoclonal antibody (lower panels). (C) Knockdown of PHLPP isoforms in normal breast epithelial cells results in increased expression of PKC. Hs578Bst (lanes 1 and 2) and MCF-10A (lanes 3 and 4) cells were transfected with control or PHLPP- specific siRNA (a combination of siRNAs for both PHLPP1 and PHLPP2 isoforms), and whole cell lysates were analyzed by Western blot using anti-PKCα, anti-PKCβII, or anti-PKCε antibodies. The expression of PKC relative to the control sample is indicated below the PKC panels.

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Chapter 5

The PHLPP Family of Phosphatases: Critical Regulators of Intracellular Signaling Pathways

Abstract

The Ser/Thr specific phosphatase PHLPP (PH domain leucine-rich repeat protein phosphatase) has emerged as an important regulator of Akt and PKC mediated intracellular signaling pathways. PHLPP controls the amplitude and duration of signaling of the Ser/Thr kinase Akt, also known as PKB, in response to various mitogenic factors. Akt is a key-signaling molecule that regulates several cellular functions including survival, proliferation, cell growth and migration. Aberrant regulation of Akt plays a causative role in many diseases including diabetes and cancer. The recently identified PHLPP downstream target, PKC, is also an important regulator of cell growth and proliferation. This Ser/Thr kinase has been implicated in the process of tumorigenesis and other diseases, such as Alzheimer's. This chapter will review the discoveries in this thesis regarding the phosphatase PHLPP and its regulation of the downstream substrates Akt and PKC. It will provide insight into the complex regulation of unique Akt isoforms by specific PHLPP isoforms, and how this dictates regulation of downstream substrates of Akt. The role of PHLPP in cellular processes, such as proliferation and apoptosis will be highlighted. Lastly, the potential role of PHLPP in various diseases including cancer and diabetes will be discussed.

With the discovery that PHLPP phosphatases are novel regulators of some AGC-type kinases, we have expanded our understanding of intracellular signaling pathways and provided new targets for therapeutic intervention (1-3). The revelation that PHLPP regulates the activity of both Akt and PKC, albeit by unique mechanisms, has shed light on how signaling by these kinases is terminated (1-3). These kinases control critical signaling nodes regulating unique biological functions from cell growth and proliferation to formation of long-term memories (4, 5). We have just begun to understand the role of these phosphatases in regulating intracellular signaling pathways, but identifying these phosphatases, as key regulators of agonist-induced Akt activity (1) will surely encourage continued study of this phosphatase family.

Akt has been identified as integral player in many diseases such as diabetes, and cancer, as well as bacterial associated illnesses (4, 6). To fully exploit the PI3K/Akt pathway as a therapeutic target it is imperative that we fully understand all signaling molecules involved in regulating this pathway. In just the past 4 years (since I joined the Newton lab) there has been an explosion in our understanding of how Akt activity is regulated in the cell and this opens the door for acute modulation of this pathway. Recent discoveries include identification of the kinase complex, mTORC2 (7-9), which regulates Ser 473 phosphorylation. This complex directly phosphorylates the turn motif (Thr 450) (10) and it remains to be determined if it directly phosphorylates the hydrophobic motif or if promotes autophosphorylation at this critical residue. There has been significant progress in our understanding of how unique Akt isoforms contribute to various diseases and this begs for drugs that will

specifically activate or inhibit specific isoforms of Akt (either directly or indirectly) associated with a specific disease (6, 11, 12). Finally we have expanded our understanding of how signaling by this kinase is terminated. The seminal discovery that PTEN dephosphorylates the 3' position of the phosphoinositides ring provided the first mechanism for shutting down activation of this pathway, which results in removal of the lipid second messenger PIP3 (13). We have now contributed to our understanding of how Akt is dephosphorylated at the hydrophobic motif, Ser 473 (1, 3).

Review of the PHLPP phosphatases.

The PHLPP phosphatase family includes three members, PHLPP1α, PHLPP1β, and PHLPP2. PHLPP1 and PHLPP2 are derived from two unique genes located at chromosome 18q21.33 and 16q22.3 respectively (1, 3). PHLPP1 and PHLPP2 possess identical domain structure with a RA domain, PH domain, leucine-rich repeats, PP2C phosphatase domain and a PDZ binding motif (1, 3). Analysis of the PH domain of PHLPP1 expressed in bacteria demonstrated that the PH domain binds phosphoinositides singularly phosphorylated at the 4', 5', bis-phosphorylated at 3' and 5' and tris-phosphorylated at 3', 4', and 5' (PIP3) positions of the phosphoinositide ring (JB, unpublished data). Further analysis needs to be performed to determine if the PH domain binds phosphoinositides in vivo and localizes to membrane domains known to contain high levels of PIP3. The PHLPP phosphatase domain is a PP2C phosphatase domain and is catalytically functional towards

synthetic substrates (para-nitrophenylphosphate (pNPP)) and protein substrates (1, 3). Kinetic analysis of the PHLPP2 phosphatase domain purified from bacteria revealed a kcat of $0.015 \pm 0.001\ s^{-1}$ and a Km of 4.13 ± 0.05 mM towards pNPP (1). The Km is comparable to other PP2C family members, but the Kcat is approximately 100-fold lower than PP2Cα. This is likely due to improper folding of the PP2C domain in bacterial cells, possibly due to absence of chaperone proteins present only in mammalian cells. Indeed, we observe an increased Kcat towards pNPP for the PHLPP2 PP2C domain expressed in insect cells (E. Sierecki, unpublished data). An alternative explanation is that the PP2C domain requires expression of the full-length protein, to attain the most catalytically competent conformation. Finally, pNPP may not be a good substrate for the PP2C domain of PHLPP since the observed rate of dephosphorylation of Akt was identical to the rate of dephosphorylation of pNPP by PP2Cα (3).

Cellular localization studies reveal that both PHLPP1 and PHLPP2 are present in the cytosolic, nuclear, and membrane fraction of cells (1). Both phosphatases are expressed in numerous cell lines examined, including brain, breast, lung, prostate, and ovarian cell lines (1, 3). Consistent with this, both phosphatases are expressed in the majority of tissues in both the mouse (unpublished data) and human (NCBI Unigene and GNF online tissue databases). Both PHLPP1 and PHLPP2 are highly expressed in the brain based on Northern blot analysis (unpublished data) and online tissue databases.

PHLPP: A play of three Akts

Akt. Akt exquisitely controls the balance between cell survival, growth and proliferation (4). Signaling molecules that promote cell survival, growth and proliferation typically bind receptors that activate the lipid kinase, phosphatidylinositol 3-kinase (PI3K) (14). Once activated PI3K generates 3'-phosphoinositide lipid second messengers, most notably phosphatidylinositol-3,4,5-trisphosphate (PtdIns P3) (15). This lipid product recruits Akt to the plasma membrane, where Akt is activated by two sequential phosphorylations: first by PDK-1 on the activation loop (Thr 308) followed by phosphorylation on the hydrophobic motif (Ser 473) (16). Additionally, Akt is phosphorylated at the turn motif, (Thr 450) and this likely occurs prior to phosphorylation at activation loop and hydrophobic motif (10). The phosphorylated species of Akt is locked in an active conformation that allows phosphorylation of downstream substrates. Phosphorylation of Akt at the hydrophobic motif is regulated by the kinase complex mTORC2, composed of rictor, mLST8, mSin1 variants, and mTOR (7-9, 17). An additional component of the mTORC2 kinase complex has recently been identified (Protor), but its role in regulating hydrophobic motif phosphorylation of Akt is unknown (18). It is unknown how mTORC2 is activated by growth factors to promote phosphorylation of Akt at Ser 473. Akt singularly phosphorylated at T308 regulates a subset of Akt substrates and additional phosphorylation at Ser473, fully activates Akt to regulate all characterized downstream substrates of Akt (7, 8). Ensuing studies revealed that both PHLPP1 and PHLPP2 are critical regulators of agonist-induced activation of Akt, as loss of either PHLPP1 or PHLPP2 results in a striking 30-fold increase in the amplitude of Akt

phosphorylation, following agonist stimulation (1). There is also a striking increase in the duration of Akt phosphorylation following agonist-induced activation of Akt (1), highlighting the importance of both of these phosphatases in regulating this pivotal kinase that controls both cellular survival and proliferation among other biological functions.

Interestingly, following agonist-stimulation of Akt, in the absence of PHLPP phosphatases, there is a dramatic increase in phosphorylation of Akt at Thr 308 in addition to Ser 473 (1). Under basal conditions PHLPP specifically regulates Akt phosphorylation at the hydrophobic motif. The increase in phosphorylation at both sites is likely due to an increase in Akt phosphorylation at Ser 473 shielding Akt from dephosphorylation at Thr 308 by other phosphatases (19). An alternative explanation is that Akt phosphorylated at Ser 473 makes a better substrate for PDK-1, as this has been observed with a phosphomimetic construct of Akt (S473D) (19). Consistent with this hypothesis, we have observed minimal increases in agonist-induced phosphorylation of Akt at Thr 308 in an Akt construct resistant to dephosphorylation by PHLPP (S473D and S473A), unpublished data. Additionally, depletion of PHLPP from cells lacking a vital component of the mTORC2 complex, mSIN1 (resulting in a lack Ser 473 phosphorylation in these cells), does not result in an increase in Thr 308 phosphorylation following stimulation with an agonist.

Akt is a crucial orchestrator of proliferation, cell survival, and cell growth (Figure 1). Akt regulates these processes by phosphorylating downstream substrates that are important components of these biological outputs (for a detailed review see

Manning and Cantley (4)). There have been over 100 described downstream substrates of the kinase Akt, however we will primarily focus on only those substrates identified to date that play a role in PHLPP-mediated Akt signaling. Akt phosphorylates the cell cycle inhibitors p21 and p27 on a Thr residue located in the nuclear localization signal to promote cytosolic sequestration of these cell cycle inhibitors and progression through the cell cycle (20-23). Another Akt substrate involved in cell cycle regulation is the E3 ubiquitin ligase HDM2 (24). Akt phosphorylates HDM2 to promote its nuclear import, and once present in the nucleus, HDM2 binds to p53 and inhibits p53-mediated transcription. HDM2 bound to p53 also promotes the nuclear export of p53 and its proteome-mediated degradation, ultimately leading to progression through the cell cycle and inhibition of apoptosis (Figure 1) (24).

Akt regulates cell survival through phosphorylation of members of the Forkhead Box O (FoxO) family of transcription factors (including FoxO1, FoxO3A and FoxO4) (4, 25). Phosphorylation of these transcription factors results in sequestration in the cytosol, mediated by binding to 14-3-3 proteins, and inhibtion of FoxO mediated transcription of pro-apoptotic proteins (Figure 1) (4). Another mediator of cell survival, which is regulated by Akt, is glycogen synthase kinase-3; phosphorylation of this substrate at Ser 9/22 results in inhibition of kinase activity, leading to inhibition of apoptosis.

A third biological output regulated by Akt is cell growth. Akt regulates cell growth through activation of the mTORC1 pathway. Akt can activate this kinase

complex by phosphorylation of two proteins involved in suppressing the activity of the mTORC1 kinase complex. The first, TSC2 (tuberin), is a tumor suppressor that forms a heterodimeric protein complex with TSC1 (hamartin), this protein complex is a GTPase activating protein (GAP) complex for the small G protein rheb (26). In the absence of TSC1/TSC2, rheb is found in an activated GTP-bound state, which is able to activate the mTORC1 complex by binding and suppressing the function of the endogenous mTORC1 inhibitor, FKP38 (27, 28) . Akt phosphorylates TSC2 inhibiting its GAP activity towards Rheb, thereby activating the mTORC1 complex (Figure 1) (29). Additionally, Akt phosphorylates PRAS40 (Proline-rich Akt substrate 40 kDa) to cause its dissociation from the mTORC1 complex, resulting in activation of this kinase complex (30). Activated mTORC1 phosphorylates 4EBP-1 causing its dissociation from eIF-4E, resulting in increased cap-dependent translation and cell growth (31). Activated mTORC1 also phosphorylates p70S6K at Thr 389, the hydrophobic motif, resulting in activation of this AGC kinase, which phosphorylates the downstream substrates eIF4B and 40S ribosomal subunit S6 (31). Additionally, phosphorylation of p70S6K causes its dissociation from eIF3 and thereby promotes protein translation, cell growth and proliferation (32). Aberrant activation of Akt can result in unregulated cell growth, proliferation, and inhibition of apoptosis, hallmarks of the tumorigenic process (33).

Depletion of endogenous Akt and PHLPP isoforms has identified unique signaling axes, whereby specific PHLPP isoforms regulate the phosphorylation state of specific Akt isoforms; these Akt isoforms in turn regulate the phosphorylation state

of specific downstream substrates (34). Depletion of endogenous PHLPP2 results in an increase in phosphorylation of the following Akt substrates: GSK-3β, TSC2, FoxO, and p27 (1). Depletion of endogenous PHLPP1 results in an increase in the phosphorylation state of many of the same Akt substrates (TSC2, FoxO and GSK-3β), but also increased the phosphorylation state of a unique set of Akt substrates including HDM2 and GSK-3α (1). Recent reports demonstrate that Akt isoforms can regulate the phosphorylation of specific downstream substrates of Akt. For example, Akt2 was reported to specifically regulate the phosphorylation of GSK-3α (35). The next step was to determine if the specific PHLPP isoforms were regulating specific Akt isoforms to account for the observed increase in phosphorylation of specific Akt substrates. To dissect the molecular mechanisms dictating this unique regulation of Akt downstream substrates, endogenous PHLPP isoforms were depleted from cells and the phosphorylation state of each Akt isoform was examined. These studies revealed that PHLPP1 regulates Akt2 and Akt3 phosphorylation at the hydrophobic motif, while PHLPP2 regulates phosphorylation of Akt1 and Akt3 at the hydrophobic motif (1).

Through depletion of endogenous Akt isoforms and monitoring the phosphorylation of downstream substrates of Akt, we begin to get a picture of how PHLPP1 and PHLPP2 regulate specific Akt isoforms to control their respective activity towards specific downstream substrates. A general conclusion from these results is that there are both specific and overlapping signaling pathways used to control the phosphorylation of downstream substrates of Akt. To elaborate further on

these novel cellular signaling pathways we will highlight pathways specific to PHLPP1 and PHLPP2. Through governing the phosphorylation of Akt2, PHLPP1 specifically controls the phosphorylation of HDM2 and GSK-3α, thus defining this specific signaling axis (Figure 2) (1). Similarly, PHLPP2 governs the phosphorylation of Akt3 to control the phosphorylation of p27. Despite both PHLPP1 and PHLPP2 constraining the phosphorylation of Akt3, only PHLPP2 depletion effects p27 phosphorylation. This suggests that cellular localization, and unique scaffolding complexes dictate this axis of regulation (1). Even in overlapping signaling pathways, there is specificity: PHLPP1 regulates Akt2 and PHLPP2 controls Akt1 to establish the phosphorylation state of TSC2 (1). Thus through depletion of endogenous PHLPP and Akt isoforms we are able to begin to understand how the cell differentially regulates the phosphorylation of downstream substrates of Akt (Figure 2).

These defined axes of signaling were also present in normal breast cells under agonist-induced conditions. Depletion of PHLPP2 from normal breast cells, following agonist stimulation, resulted in a robust increase in p27 phosphorylation. This increase could be rescued by the simultaneous knockdown of the Akt3, confirming this molecular pathway of PHLPP2-Akt3-p27 is intact under both basal and growth factor stimulated conditions (1). This proof of principle experiment is the first step in a systems biology approach to begin to understand the complex network of cellular signaling regulated by PHLPP. These signaling axes have been deduced following a reductionist approach that ultimately results in disassembling the engine known as the

human cell down to its very basic components of nuts and bolts. However, understanding what the very basic components of an engine are does not provide information regarding how the engine works. Only when we start to piece this complex puzzle together, by depleting the cell of multiple components, do we begin to get a clearer picture of the intricate signaling mechanisms driving various functions of the cell.

There is another level of regulation of downstream substrates of Akt dictated by phosphorylation state of Akt at the activation loop and hydrophobic motif. Recent studies have reported that Akt phosphorylated at Thr 308 is active towards a subset of downstream substrates of Akt, including GSK-3 and TSC2 (7, 8). These studies examined mouse embryonic fibroblasts lacking components of mTORC2 and were therefore deficient of Ser 473 phosphorylation. Stimulation with agonists causes increases in Thr 308 phosphorylation as well as increases in phosphorylation of downstream substrates (7, 8). They did not observe increases in phosphorylation of FoxO transcription factors. These studies add another level of complexity to the acute regulation of downstream signaling of Akt where phosphorylation state combined with isoform specificity contribute to regulation of downstream signaling and ultimately biological output. However, these studies did not verify that inhibition Akt prior to growth factor stimulation abrogates phosphorylation of downstream substrates to verify that other kinases are not compensating for lack of a catalytically competent Akt. p70S6K has been demonstrated to phosphorylate Akt substrates, such as GSK-3α/β, in cells lacking activated Akt (36). A similar mechanism could account for

phosphorylation of these substrates in mTORC2 deficient cells. However, we observe only a modest increase in phosphorylation of GSK-3 in mSIN1 deficient cells, pretreated with PI3K inhibitor LY294002, following agonist stimulation, suggesting Akt singularly phosphorylated at Thr 308 is still controlling a majority of the phosphorylation towards this substrate. Differences in our observation that Akt singularly phosphorylated at Thr 308 results in a decrease in GSK-3 and TSC2 phosphorylation may be due to differences in cell types and organisms. The mTORC2 deficient cells are early mouse embryonic fibroblasts, while our results were performed in normal breast cells or lung cancer cells, and this could account for the observed differences.

PKC. PKC is another member of the AGC family of kinases with 10 isozymes present in mammalian cells (37). The PKC family members are grouped into three distinct families based on the presence or absence of diacylglycerol (C1) and Ca^{+2} (C2) binding domains. Similar to Akt, PKC is a substrate for the kinase PDK-1, which phosphorylates PKC at the activation loop (Thr 497, PKCα) (37). This triggers two intramolecular auto-phosphorylations at the turn motif and hydrophobic motif, which locks PKC in a catalytically competent conformation (37). We have recently discovered that the PHLPP phosphatases dephosphorylate the hydrophobic motif of PKC, resulting in degradation of PKC (2). Depletion of the PHLPP phosphatases results in an increase in PKC phosphorylation at the hydrophobic motif (Ser657 PKCα) and increased PKC protein levels (2).

Interestingly, cells deficient of the mTORC2 complex, have decreased PKC

phosphorylation at the hydrophobic motif, suggesting this complex is contributing to phosphorylation of PKC at this site (7). This suggests the PHLPP phosphatases act in an opposing manner to the mTORC2 complex. Further studies should elaborate on the interplay between these signaling molecules.

Other Signaling Pathways Regulated by PHLPP. Other potential substrates of PHLPP include AGC kinase family members such as p70S6K, SGK or p90RSK. Overepxression studies suggest that PHLPP2 does not regulate either p70S6K or p90RSK (1), however under these conditions the phosphatase may be mislocalized and unable to regulate potential downstream substrates. Depletion of either PHLPP1 or PHLPP2 results in an increase in p70S6K phosphorylation at the hydrophobic motif, Thr 389 (1). p70S6K could be a direct target of PHLPP, however this increase in Thr 389 phosphorylation could be explained by an increase in Akt activity. Increased Akt activity would result in an increase in TSC2 phosphorylation, activating Rheb, leading to the activation of mTORC1 (additionally Akt phosphorylates and causes dissociation of the direct mTORC1 inhibitor PRAS 40), which phosphorylates p70S6K at Thr 389 (38). Further studies need to be performed examining the regulation of p70S6K in the absence of PHLPP, where Akt activation is inhibited. For example, starving cells and then exposing them to amino acids should activate mTORC1, independent of Akt activation.

Cellular Processes Regulated by PHLPP

Proliferation. Proliferation is a complex cellular process involving increases in protein translation, genome duplication, and ending with cytokinesis resulting in

two identical cells sharing equal cellular components. This process is intricately regulated by signaling pathways including the Raf-MEK-ERK pathway and PI3K-Akt pathway. As alluded to earlier, one mechanism by which Akt directly regulates this process is through phosphorylation of the cell cycle inhibitors p27 and p21. In addition to this direct mechanism of cell cycle regulation, Akt indirectly regulates cell cycle progression though inhibition of GSK-3α/β kinase activity. GSK-3α/β regulates the cell cycle by phosphorylating Cyclin D and Cyclin E, targeting these proteins for proteosomal degradation. Inhibition of GSK-3α/β by Akt results in stabilization of these cell cycle proteins and progression from G1 to S phase of the cell cycle (4). Consistent with Akt regulating cell cycle progression and proliferation, PHLPP is critical regulator of this biological process (1). Loss of endogenous PHLPP results in a decrease in the G1/S ratio, suggesting cells are entering the cell cycle at an increased rate (1). Consistent with these data, depletion of PHLPP results in an increase of BrdU incorporation; indicating cells are replicating their genomes at increased rate (1). Overexpression of PHLPP caused an increase in the G1/S ratio confirming that PHLPP is a regulator cell cycle progression (1). Lastly, stable cells lines expressing PHLPP1α have decreased cell numbers compared to vector control, suggesting the cells could be proliferating at a decreased rate, but this could also be due to an increase in apoptosis (3). These data confirm that PHLPP is newly identified inhibitor of cell cycle progression and proliferation.

The exact molecular mechanism defining cell cycle regulation by the PHLPP phosphatases has not been elucidated, but it was observed that PHLPP2 is a more

potent regulator of cell cycle progression. Depletion of PHLPP2 resulted in a more robust increase in BrdU incorporation and a greater decrease in the G1/S ratio. Both PHLPP isoforms regulate the phosphorylation state of GSK-3β; so while this pathway may contribute PHLPP-mediated cell cycle regulation, it would not contribute to the observed differences. These differences are more than likely due to PHLPP2 specific regulation of the cell cycle inhibitor p27 through the Akt3 isoform. PHLPP1 may regulate the cell cycle through an alternative pathway involving HDM2 phosphorylation, (24). This would suggest that direct phosphorylation of p27 results in a more pronounced effect on the cell cycle compared to HDM2 regulation by Akt.

Lastly we would like to highlight the potential of PHLPP to regulate cellular proliferation through the Raf-MEK-ERK pathway. Overexpression of PHLPP1β suppresses the activation of this pathway and it is likely that this may contribute to inhibtion of cell cycle progression observed in cells when PHLPP is overexpressed (5, 39). It will be interesting to determine the contribution of the MAPK pathway to the proliferative phenotype.

Apoptosis. The process of programmed cell death is induced when cells are damaged beyond repair or have reached their finite lifespan, and is a mechanism to maintain tissue homeostasis (40). Apoptosis is triggered by cellular stresses, DNA damage, and is an immune response to clear infected cells (40). Akt is a well-characterized regulator of cell survival and does so by phosphorylating downstream substrates such as bad and FoxO family of transcription factors (4). Akt phosphorylates Bad at Ser 136 leading to 14-3-3 binding, which prevents it from

binding to anti-apoptotic proteins Bcl-X_L and Bcl-2 at the mitochondrial membrane (41). PHLPP regulates apoptosis primarily through regulating Akt phosphorylation at the hydrophobic motif. Overexpression of PHLPP causes increased apoptosis in both lung and breast cancer cell lines (1, 3). Importantly, this effect can be rescued with a phosphomimetic construct of Akt (S473D) that is resistant to regulation by PHLPP (1, 3). Interestingly this is not a complete rescue, suggesting other downstream targets of PHLPP play a role in PHLPP-mediated induction of apoptosis.

PHLPP also plays a role in promoting apoptosis in response to genotoxic agents, such as etoposide, a topoisomerase II inhibitor. Depletion of PHLPP inhibits etoposide-induced apoptosis confirming the role of PHLPP in regulating cellular survival. These data also suggest that PHLPP may play a role in chemotherapeutic resistance, and inhibition of PHLPP activity could be a mechanism, by which a cancer cell becomes resistant to chemotherapy. Indeed, Akt has already been demonstrated to promote resistance to chemotherapy, and loss of PHLPP regulation should enhance this effect (42).

Diseases Associated with PHLPP

Cancer. The PI3K-Akt pathway is a central node in determining cellular fate. Hyperactivation of this pathway increases cell survival, growth and proliferation, hallmarks of the tumorigenic process (43). Somatic mutations resulting in constitutive activation of this pathway are well documented and defined as a causal mechanism driving tumorigenesis. This pathway is activated in cancer by a number of

mechanisms, including amplification or gain of function mutations in upstream receptor protein tyrosine kinases (RPTKs) (14, 44, 45), activating mutations in PI3K and Akt (11, 46), and loss of function mutations in the regulatory phosphatase PTEN (phosphatase and tensin homolog on chromosome ten) (47). Given the importance of this pathway in tumorigenesis, it is important to elucidate if the PHLPP phosphatases are involved in this process.

In addition, PKC is the receptor for tumor-promoting phorbol esters, strongly implicating this kinase in tumorigenesis (37). PKC is also somatically mutated in many cancers, however the functional consequences of these mutations have not been determined (48). Providing further rationale that PHLPP phosphatases could be involved in tumorigenesis, both genes are located at chromosomal loci frequently lost in cancer, as highlighted earlier.

To determine if the PHLPP isoforms are mutated in cancer we have sequenced the open reading frames of both genes in various cancer cell lines. These sequencing efforts have revealed a functional polymorphism in the PHLPP2 phosphatase that results in Leu to Ser change at codon 1016 in the PP2C phosphatase domain. This functional polymorphism reduces the basal activity of the phosphatase towards Akt and PKC, but it remains to be determined if it plays a role in breast cancer. We have also identified a somatic mutation in the PH domain of the PHLPP2 phosphatase. We have not observed any somatic mutations or polymorphisms in the PHLPP1 gene. We have yet to determine the functional consequences of the PH domain mutation (Q190H), but there is precedence for a PH domain mutation playing a role in cancer; a

mutation in the PH domain of Akt results in membrane localization and constitutive activation of the kinase. It is possible that the mutation in the PH domain of PHLPP may conformationally control PHLPP (for example, by potentially increasing autoinhibition of the phosphatase domain), alter the subcellular location of PHLPP, or affect its interaction with binding partners.

Given the specific regulation of Akt isoforms by PHLPP isoforms it will be important to determine if there is a unique contribution of each PHLPP isoform to the process of tumorigenesis. The role of each Akt isoform in cancer initiation and progression is complex and somewhat contradictory. For example, Akt1 inhibits both invasion and cellular migration in breast cancer cell lines and immortalized MCF-10A cells, (49, 50), but Akt1 has also been reported to promote tumorigenesis and metastasis in mice expressing ErbB2 as well as promoting migration in mouse endothelial tumor cells (51). Consistent with the latter result, Akt1 knockout mice have delayed tumor onset when crossed with mice expressing the oncogenes ErbB2 or polyoma middle T antigen (52). These studies illustrate the complexity of Akt1's role in cancer and provide evidence that Akt1 can be both a tumor suppressor and an oncogene. Activation of Akt2 leads to increased migration, invasion and metastasis in cell culture systems (50). Surprisingly Akt2 knockout mice crossed with mice expressing ErbB2 or polyoma middle T antigen have an increased rate of tumor development, suggesting Akt2 may inhibit tumor progression (52). Again, these observations highlight the complexity in understanding the contribution of each Akt isoform in tumorigenesis. In summary, these data suggest that PHLPP1 and PHLPP2

could be both tumor suppressors and oncogenes.

Unlike PTEN, which is somatically mutated in many cancers resulting in loss of function or expression, the PHLPP phosphatases are expressed and functional in the majority of cancer cell lines examined (47). This presents a unique opportunity where the PHLPP phosphatases could make universal targets for the treatment of cancer. If small molecule compounds can be discovered that result in activation of these phosphatases this could result in loss of activation of both PKC and Akt, and ultimately lead to tumor regression. To this end it has recently been reported that lanthanum nitrate treated rats have a 23-fold increase in the expression of PHLPP2 (48). A compound such as this may present exciting opportunities for cancer treatment. It would be interesting to test if other lanthanum based compounds could increase the expression of PHLPP2, such as lanthanum carbonate (fosrenol), which is FDA approved and used for the treatment of renal failure. Many lanthanum-based compounds cause apoptosis and inhibit proliferation of cancer cells (53, 54), by unknown mechanisms. One possibility is that these compounds may increase PHLP2 expression. Given the paradigm shift in drug discovery from specific inhibitors to pan inhibitors that shut down multiple signaling pathways implicated in cancer (55), discovery of a small molecule that activates or increases expression of PHLPP should result in the inactivation of multiple cancer-related signaling pathways.

Metabolic Disorders. Another function of Akt signaling is to stimulate glucose uptake in response to insulin (4). Akt2 is the main isoform responsible for glucose uptake, evidenced by the Akt2 knockout mouse, which has a type II diabetic

phenotype. Additionally, Akt2 is the main isoform detected in insulin responsive tissues (56). Loss of Akt2 results in insulin resistance, and hyperglycemia due to loss of glucose uptake (56). Consistent with these results overexpression of an inhibitor of Akt, tribbles (TRB3), promotes hyperglycemia in mice (57). Akt stimulates glucose uptake by promoting translocation of Glut4 to the plasma membrane. Direct inhibtion of PHLPP1 should stimulate Akt2 activity and could be a potential therapeutic target for the treatment of type II diabetes.

The Future of PHLPP Research

We have just begun to understand how the PHLPP phosphatases contribute to the intricate cell signaling networks that dictate cellular function. Much work lies ahead to fully decipher the role that these phosphatases play in normal tissue homeostasis as well pathophysiological states. With the knockout mice for PHLPP1 and PHLPP2 currently being generated we should begin to get a picture of how these phosphatases contribute to organismal development and disease. Importantly, a PHLPP1/PHLPP2 double knockout mouse should be generated to determine the phenotypes of this mouse. One would hypothesize that these mice should show similar phenotypes to knockin mice expressing active Akt. Given the observed isoform-specific regulation of Akt, will the PHLPP1 knockout mouse demonstrate hyperinsulinemia due to hyperactivation of Akt2? Will the PHLPP2 mice demonstrate an increased rate of hyperplasia? Answers to many exciting questions will be addressed with these mice.

Future studies should also be aimed at elucidating novel targets of the PHLPP phosphatases. Given that there are more than 400 Ser/Thr kinases, but less than 40 Ser/Thr phosphatases, it can be deduced that these phosphatases will have multiple substrates (58). Exciting challenges lie ahead to decipher the full spectrum of signaling pathways regulated by PHLPP, but a clear picture of how a cell operates is crucial to our understanding of signal transduction and how these pathways can go awry in various diseases.

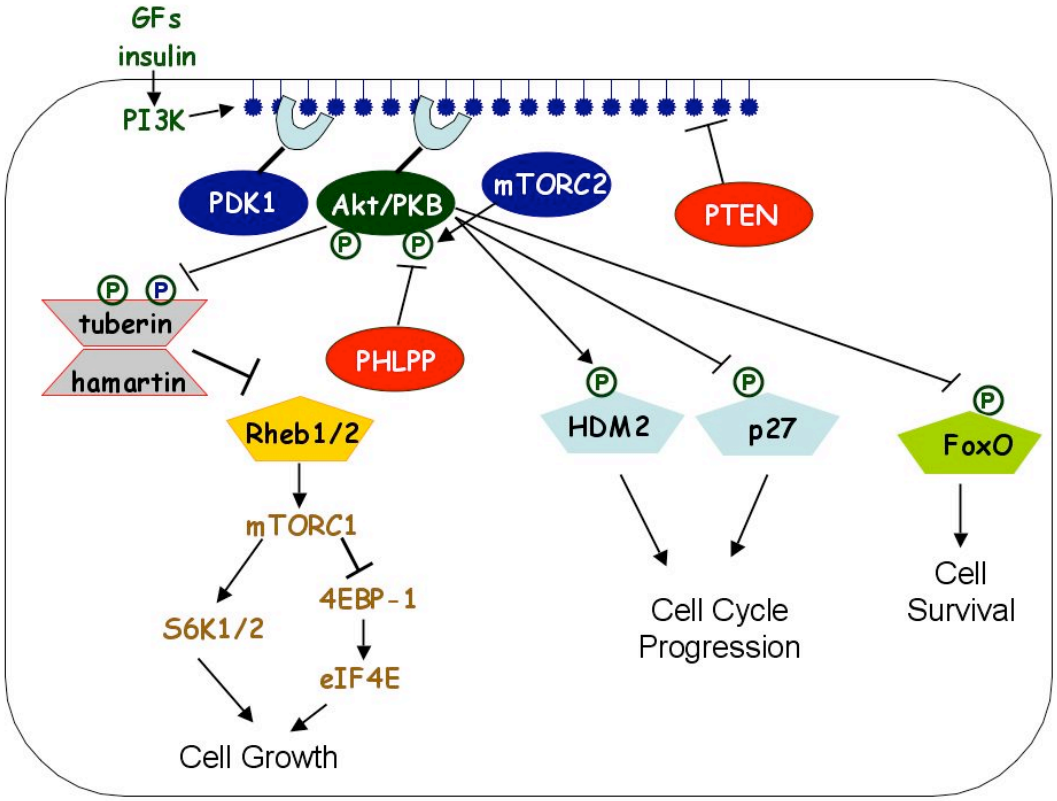

Figure 5.1: PHLPP regulates the activity of Akt downstream substrates to promote cell growth, proliferation and inhibition of apoptosis. Akt controls the phosphorylation state of the GTPase activating complex (TSC1/TSC2). Phosphorylation of TSC2 by Akt results in activation of Rheb, leading to the activation of mTORC1 and promotion of cell growth. Akt regulates cell cycle progression by directly phosphorylating HDM2 and p27, promoting progression through the cell cycle. Lastly, Akt inhibits FoxO mediated transcription through phosphorylation, which causes cytosolic sequestration of these transcription factors and inhibits their transcription of proapoptotic proteins. PHLPP antagonizes Akt function, by inhibiting the activation of the kinase resulting in induction of apoptosis, and inhibtion of both cell cycle progression and cell growth.

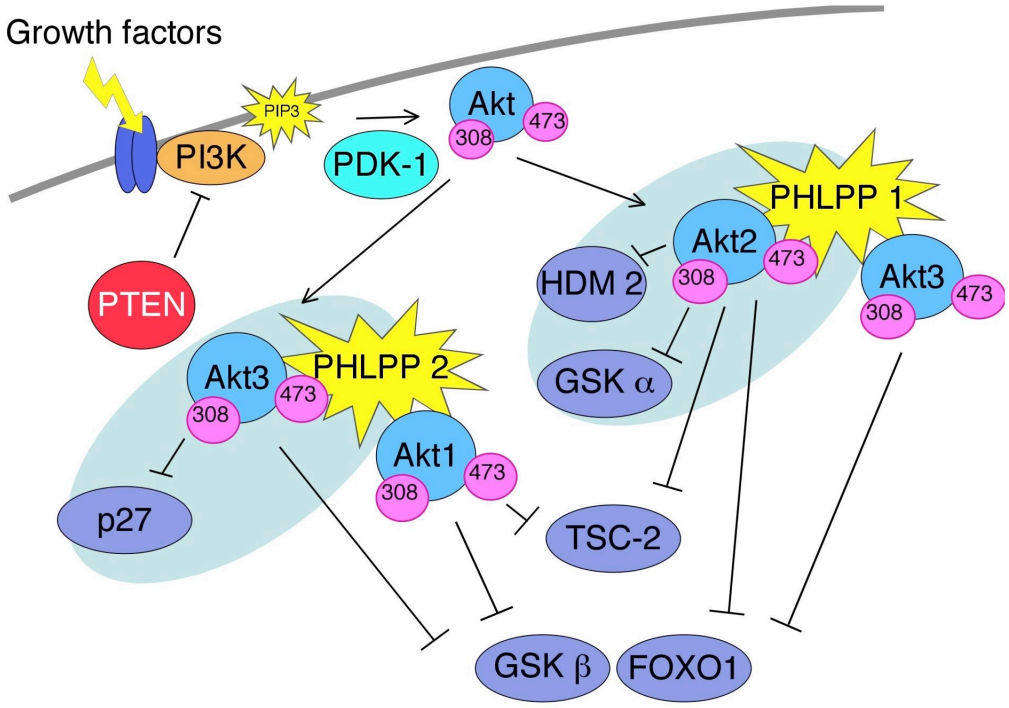

Figure 5.2: PHLPP1 and PHLPP2 selectively control the phosphorylation state of specific Akt isozymes, which in turn control the phosphorylation of specific substrates. Following mitogen stimulation, Akt is phosphorylated on the activation loop (Thr308 in Akt1) by PDK-1, an event that triggers phosphorylation on the hydrophobic motif (Ser473 in Akt1). Akt1, Akt2, and Akt3 phosphorylate both isoform-specific (HDM2, p27) and shared (GSK-3β) substrates. Specific complexes (light blue ovals) of PHLPP1 or PHLPP2 with Akt1, Akt2, or Akt3 allow PHLPP isoforms to differentially control the amplitude of Akt signaling towards downstream substrates.

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