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Loss of PHLPP expression in colon cancer: Role in proliferation and tumorigenesis

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Abstract

PHLPP (PH domain Leucine-rich-repeats Protein Phosphatase) represents a family of novel Ser/Thr protein phosphatases. Two highly related isoforms in this family, PHLPP1 and PHLPP2, have been identified to serve as negative regulators of Akt and protein kinase C (PKC) by dephosphorylating the kinases directly. In this study, we examined the expression pattern of both PHLPP isoforms in colorectal cancer specimens and the adjacent normal mucosa using immunohistochemical (IHC) staining. We found that the expression of PHLPP1 or PHLPP2 isoform was lost or decreased in 78% and 86% of tumor tissues, respectively. Stable overexpression of either PHLPP isoform in colon cancer cells decreased the rate of cell proliferation and sensitized the cells to growth inhibition induced by the phosphoinositide-3-kinase (PI3K) inhibitor, LY294002, whereas knockdown of either PHLPP isoform by shRNA promoted the proliferation of DLD1 cells. In addition, we demonstrated that the PHLPP-mediated growth inhibition in colon cancer cells was largely rescued by overexpression of a constitutively active Akt. Moreover, re-expression of either PHLPP isoform in HCT116 cells inhibited tumor growth *in vivo*. Taken together, our results strongly support a tumor suppressor role of PHLPP in colon cancer.

Keywords

PHLPP; colon cancer cells; Akt; protein kinase C; and tumorigenesis

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Introduction

Dysregulation of signaling pathways mediated by phosphoinositide-3-kinase (PI3K) and PKC have been implicated in the development of colorectal cancer (Gokmen-Polar et al., 2001; Samuels et al., 2005; Samuels et al., 2004). Serving as a major regulator downstream of PI3K, Akt promotes tumor growth by promoting cell proliferation and inhibiting apoptosis (Manning & Cantley, 2007). In addition, upregulation of PKC βII contributes to tumorigenesis of colon cancer by inducing hyper-proliferation (Gokmen-Polar et al., 2001; Murray et al., 1999). Both Akt and PKC belong to the AGC kinase superfamily, and they share three highly conserved phosphorylation sites namely the activation loop, turn motif, and hydrophobic motif sites (Newton, 2003). Phosphorylation of Akt is required for the activation of the enzyme following activation of PI3K, whereas phosphorylation of PKC does not control the enzyme activity directly rather it is constitutive and necessary for maintaining the stability of the protein (Newton, 2003). Since the activity and the total protein expression of Akt and PKC are controlled by phosphorylation, dephosphorylation leads to effective signaling termination by either inactivating the enzyme directly or by decreasing the functional lifetime of the protein. However, the phosphatases responsible for dephosphorylating the AGC kinases are largely elusive.

Recently, a family of novel protein phosphatases, namely PHLPP, have been identified as the phosphatases for Akt and PKC. Two isoforms, PHLPP1 and PHLPP2, found in this family, share their ability to dephosphorylate both Akt and PKC directly (Brognard et al., 2007; Gao et al., 2005). Specifically, overexpression of PHLPP results in a decrease of Akt activity and an increase of apoptosis in non-small cell lung cancer (H157) and breast cancer cells, while knockdown of PHLPP displays an opposite effect (Brognard et al., 2007; Gao et al., 2005). However, since phosphorylation of PKC is required for maintaining the protein stability, PHLPP-mediated dephosphorylation dictates the expression level of PKC and knockdown of PHLPP delays phorbol ester-induced PKC downregulation (Gao et al., 2008). Thus, by controlling the phosphorylation status of Akt and PKC, PHLPP plays an important role in regulating the duration and amplitude of signals evoked by these kinases.

Several lines of evidence suggest that PHLPP functions as a tumor suppressor. For example, it has been shown that overexpression of PHLPP1 in a glioblastoma cell line inhibits the tumor growth in xenografted nude mice (Gao et al., 2005), and decreased expression of PHLPP has been linked to the metastatic potential of 21T breast cancer cells (Qiao et al., 2007). However, a direct association of PHLPP and human cancers has not been established. In the present study, we aimed to elucidate the functional connection of PHLPP to colon cancer. Furthermore, the ability of both PHLPP isoforms in antagonizing tumor growth was determined *in vivo*.

Results

Loss of PHLPP expression in colorectal cancer patient specimens

To explore the link between PHLPP and human colorectal cancer, we examined the potential loss of PHLPP expression in colorectal cancer specimens. Colon cancers from both

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the left and the right side and the adjacent normal mucosa were analyzed for expression of PHLPP. The expression of both PHLPP isoforms was the highest in the surface epithelium of normal colonic mucosa, but largely excluded from the bases of the crypts (Fig. 1a and 1b). Interestingly, PHLPP1 was concentrated at the lateral membrane of the epithelial cells (Fig 1a), while PHLPP2 has mixed cytoplasmic and nuclear expression (Fig 1b). The specificity of the PHLPP antibodies was confirmed by including the blocking peptides in the IHC staining. The quantitative results based on a four-tier scoring system revealed that the expression of either PHLPP1 or PHLPP2 was lost or decreased in the cancer samples (median PHLPP1 and PHLPP2 staining score = 1.0) relative to adjacent normal colonic mucosa (median PHLPP1 and PHLPP2 = 3.0) ($p<0.0001$). The detailed statistics of the IHC staining results are summarized in Table 1. As shown in Figure 1c, the majority of the normal tissues showed high expression of PHLPP1 and PHLPP2 (100% and 96%, respectively). In marked contrast, the expression of PHLPP1 or PHLPP2 was significantly lost or decreased in 78% and 86% of tumor tissues, respectively ($p<0.0001$). Interestingly, there was a significant positive correlation of intensity staining score between the two isoforms ($r=0.75$, $p<0.0001$, Table 2), suggesting that loss of both PHLPP isoforms was commonly observed in the same patient. Therefore, the two PHLPP isoforms may function together to inhibit tumorigenesis. Taken together, these findings suggest that loss of PHLPP expression occurs at a high frequency in colorectal cancers strongly supporting a tumor suppressor role of PHLPP.

Overexpression of PHLPP inhibits proliferation of colon cancer cells

To address the functional importance of maintaining PHLPP expression in colon cancer cells, we first analyzed the basal PHLPP expression and Akt phosphorylation in three colon cancer cell lines. As shown in Figure 2a, the endogenous expression of both PHLPP isoforms was the highest in DLD1 cells. Interestingly, the phosphorylation of Akt was lower in DLD1 compared to that of HCT116 and HT29 cells. Note that there are two splicing variants of the PHLPP1 gene with molecular mass of 140 and 190 kDa expressed as reported previously (Brognard et al., 2007). The longer form (PHLPP1β) contains the entire coding sequence of the shorter form (PHLPP1α) with an additional 5′ extension. The two PHLPP1 isoforms are expected to have similar phosphatase activity as the majority of the protein sequences are shared. However, the cloning of PHLPP1β cDNA has not been reported to date. Here, we focused our studies on the functional effect of PHLPP1α [we referred to it as PHLPP1 to remain consistent with the nomenclature used previously (Brognard et al., 2007; Gao et al., 2005)].

Since both HCT116 and HT29 cells have reduced expression of PHLPP, we established stable cell lines overexpressing either PHLPP isoform and investigated the functional effect of re-introducing PHLPP into these cells. To determine the rate of proliferation, the numbers of cells were counted daily for 4 days. Overexpression of either PHLPP isoform significantly slowed the rate of proliferation in both HCT116 and HT29 stable cells lines. Specifically, the numbers of cells were reduced to 45% and 50% at day 4 in HCT-P1 and HCT-P2 cells, and 31% and 42% in HT29-P1 and HT29-P2 cells compared to the control cells, respectively (Fig. 2b). To further determine whether PHLPP inhibits cell proliferation by interfering with the cell cycle, we examined the cell cycle parameters of HCT116 stable

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cells using FACS analysis, and the representative results are shown in Figure 2c. Overexpression of PHLPP1 resulted in an increase in the percentage of cells in the G2 phase suggesting a potential block of the G2/M transition in the cell cycle. Interestingly, overexpression of PHLPP2 induced a G1 arrest as measured by increased G1/S ratio but had no effect on the numbers of G2 cells (Fig. 2d). In addition, expression of either PHLPP isoform had a more pronounced effect under the serum-starved condition (–S). These cancer cells are known to be resistant to serum starvation due to lack of ability to shut down of growth signals completely, however, they are able to turn off those signals more effectively in the presence of PHLPP. Ovexpression of PHLPP alone was unable to induce a significant amount of apoptosis as indicated by the absence of cell population in the sub-2N fractions (Fig. 2c). Furthermore, we tested the synergistic effect of PHLPP expression on cell proliferation in the presence of the PI3K inhibitor, LY294002. Treating cells with LY294002 inhibited the proliferation of HCT-Con cells in a concentration-dependent manner. Overexpression of either PHLPP isoform significantly enhanced the inhibitory effect of LY294002 at 12 and 16 μM (Fig. 2e), suggesting that the level of PHLPP expression in colon cancer cells is an important factor in determining the sensitivity to PI3K inhibition. Taken together, these results suggest that both PHLPP isoforms inhibit cell proliferation primarily by interfering with the different cell cycle checkpoints in colon cancer cells.

PHLPP modulates both Akt and PKC pathways in colon cancer cells

It has been reported previously that PHLPP serves as a phosphatase for both Akt and PKC. We next determined which pathway is regulated by PHLPP in colon cancer cells. To assess the effect of PHLPP on Akt signaling, the phosphorylation of Akt and a number of Akt substrates were examined in stable HCT116 and HT29 cells. Since PHLPP controls the level of PKC expression, the total PKC expression was analyzed as well. In HCT116 stable cells, Akt phosphorylation at the Ser473 site was decreased significantly by ~70% in both PHLPP1 and PHLPP2 overexpressing cells, while the Thr308 site was only slightly affected (Fig. 3a and 3b). This is consistent with the notion that PHLPP prefers the hydrophobic motif site (Ser473) on Akt (Brognard et al., 2007; Gao et al., 2005). In addition, dephosphorylation of Akt substrates including TSC2 (at an inhibitory phosphorylation site) and p27 was detected in HCT-P1 and HCT-P2 cells (Fig 3a and 3b). Because overexpression of PHLPP affects the cell cycle as shown in Figure 2, the total protein expression of p27 was analyzed as well. The results showed that the expression level of p27 was increased by ~50% in both HCT-P1 and HCT-P2 cells (Fig. 3a and 3b). In contrast, phosphorylation of another Akt substrate, GSK3 (including both α and β isoforms), was not altered in PHLPP expressing cells. Furthermore, since elevation of PKC βII has been implicated in colon cancer carcinogenesis, we determined whether the expression of PKC βII was modified by PHLPP. Indeed, the expression of PKC βII was reduced by ~30% in both HCT-P1 and HCT-P2 cells (Fig. 3a and 3b). Serving as a control, the level of PKC ζ, a PHLPP-insensitive PKC isozyme [due to lack of phosphorylation in the hydrophobic motif (Gao et al., 2008)], was not altered by PHLPP expression.

To confirm the results obtained in HCT116 cells, we examined the effect of PHLPP overexpression in HT29 stable cells. Similarly, dephosphorylation of Akt and its substrates

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TSC2 and p27, as well as increased expression of p27, were readily detected in HT29-P1 and HT29-P2 cells. In addition, downregulation of PKC βII but not PKC ζ was observed (Fig. 3c and 3d). Moreover, we found that the mRNA level of p27 and PKC βII was not altered by overexpression of PHLPP (Supplemental Fig. 1) suggesting a post-transcriptional modulation mediated by PHLPP. Collectively, these data indicate that overexpression of PHLPP selectively regulates Akt and a subset of Akt substrates, as well as altering the expression of PKC βII in colon cancer cells.

PHLPP-mediated growth inhibition is rescued by overexpressing an active mutant of Akt

Since overexpression of PHLPP negatively regulates both Akt and PKC as shown in Figure 3, we performed rescue experiments to dissect the relative contribution of each pathway. If PHLPP-induced inhibitory effect was mediated through Akt, overexpression of a PHLPP-resistant Akt (phospho-mimetic mutation at Ser473) should release the cells from growth inhibition. Because the PI3K/Akt pathway is basally activated due to the existence of a mutant PIK3CA allele in HCT116 cells (Samuels et al., 2005), overexpression of Akt/S473D only resulted in a moderate increase (∼15%) of cell proliferation in control cells. However, the rate of proliferation was increased by ∼2-fold in HCT-P1 and HCT-P2 cells upon Akt/S473D expression (Fig. 4a). Similar experiments were performed to examine whether PHLPP overexpressing cells could be rescued by PKC βII. Overexpression of PKC βII resulted in a ∼30% increase in cell numbers in HCT-Con cells (consistent with the role of PKC βII in promoting proliferation), however, only a 10-15% increase of cell numbers was observed in HCT-P1 and HCT-P2 cells (Fig. 4b). Collectively, these data suggested that the inhibitory effect of PHLPP was largely explained by PHLPP-mediated inactivation of Akt, as more than two-thirds of the inhibition was released by Akt/S473D expression. On the other hand, although overexpression of PHLPP induces downregulation of PKC βII, it has little contribution under our experimental conditions.

Functional effect of knocking down endogenous PHLPP in colon cancer cells

To examine the effect of knocking down endogenous PHLPP in colon cancer cells, we infected DLD1 cells (which have the highest basal PHLPP expression) with lentivirus-based shRNAs targeting PHLPP1 or PHLPP2. Two different targeting constructs were chosen for each PHLPP isoform (sh-P1-97 and sh-P1-94 for PHLPP1; sh-P2-60 and sh-P2-57 for PHLPP2). A control virus was used to create the control cell lines. The knockdown efficiency was around 60-80% for PHLPP1 and 50-90% for PHLPP2 (Fig. 5a). In the stable knockdown cells, an increase of PKC βII expression was consistently observed, while the level of PKC ζ was unchanged (Fig. 5a). To determine the effect of PHLPP knockdown on Akt phosphorylation, the stable knockdown cells (sh-P1-97 and sh-P2-60 were chosen because the knockdown efficiency was higher) were subjected to different growth conditions (Fig. 5b). The phosphorylation of Akt was approximately 1.5-2 fold higher in the PHLPP knockdown cells compared to the control cells under all conditions tested confirming the phosphatase function of endogenous PHLPP for Akt in colon cancer cells. Furthermore, we examined the functional effect of knocking down PHLPP by measuring the rate of cell proliferation. Both PHLPP1 and PHLPP2 knockdown cells proliferated at a significant faster rate compared to the control cells (Fig. 5c). Specifically, the numbers of cells were 53% and 66% higher than those of the control cells at day 4 for sh-P1-97 and sh-P2-60, or 40% and

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34% higher for sh-P1-94 and sh-P2-59, respectively. Taken together, our results underscore the importance of maintaining the level of PHLPP expression as PHLPP plays a critical role in keeping a tight control of growth signals in colon cancer cells.

Overexpression of PHLPP inhibits tumor growth in xenografted nude mice

To examine whether increased PHLPP expression in colon cancer cells affects their tumorigenic ability *in vivo*, we injected HCT-Con, HCT-P1, and HCT-P2 cells subcutaneously into the nude mice and monitored tumor growth. HCT-Con cells formed tumors effectively, and the tumor size reached approximately 400 mm^3 within 28 days. In marked contrast, the ability of HCT-P1 and HCT-P2 cells to form tumors was significantly reduced over the same period (Fig. 6a). Only small tumors with size less than 100 mm^3 were detected at the end of the experiments (Fig. 6a). To determine whether PHLPP suppressed tumor growth by decreasing the rate of proliferation, we measured the numbers of Ki-67 positive cells (proliferating cells) in the tumor sections (Fig. 6b). The results revealed that the numbers of cells stained positive for Ki-67 were significantly reduced by 44% and 55% in tumors formed by HCT-P1 and HCT-P2 cells, respectively. Furthermore, the levels of PHLPP expression and Akt phosphorylation in the tumor sections were analyzed using IHC staining. A modest increase in PHLPP expression was detected in the corresponding tumor sections, whereas the phosphorylation level of Akt was markedly decreased in tumors derived from HCT-P1 and HCT-P2 cells compared to that of HCT-Con cells (Fig 6c). Taken together, the *in vivo* experiments demonstrate that overexpression of either PHLPP isoform inhibits tumorigenesis of colon cancer cells in nude mice, and this inhibition is largely attributed to PHLPP-mediated negative regulation of cell proliferation.

Discussion

Controlling the balance of protein phosphorylation is one of the most important defense mechanisms provided by protein phosphatases to prevent aberrant hyperactivation of signaling in cells. Our studies here focused on elucidating the tumor suppressor function of two closely related protein phosphatases, PHLPP1 and PHLPP2, in colon cancer. We found that loss of PHLPP expression is associated with 70-80% of patient samples. Overexpression of either PHLPP in colon cancer cells decreases the rate of cell proliferation, sensitizes the cells to PI3K inhibitor, and antagonizes the tumorigenesis process in nude mice. While both Akt and PKC are modulated by PHLPP, the rescue experiments indicate that PHLPP-mediated growth inhibition is largely due to its negative regulation of Akt signaling.

The tumor suppressor function of PHLPP has been suggested based on the initial findings that both PHLPP isoforms serve as negative regulators of Akt (Brognard et al., 2007; Gao et al., 2005). In this study, we provide the first direct evidence linking PHLPP to colon cancer. Intriguingly, we did not find an inverse correlation between loss of PHLPP expression and cancer stage. It is possible that loss of PHLPP expression provides growth advantage for tumor cells at early stages, however, PHLPP may play a different role in cancer metastasis during the late stage of tumor, as this is the case for Akt1 and Akt2 in the tumorigenesis and metastasis processes of breast cancer (Maroulakou et al., 2007; Yoeli-Lerner et al., 2005). Interestingly, the highest expression of both PHLPP isoforms was found in the fully

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differentiated cells in the upper crypts of the normal colonic mucosa. In agreement with our findings, it has been shown that mRNA of the PHLPP2 gene is preferentially localized to the villi of the small intestine using microarray analysis (Kosinski et al., 2007; Saaf et al., 2007). This differential expression pattern of PHLPP is consistent with its role in inhibiting cell proliferation and promoting apoptosis, as the epithelial cells lose their ability to proliferate and begin to undergo apoptosis as they reach the tip of the villus. Future studies with a larger and more comprehensive sample set are required to fully address whether loss of PHLPP expression is tumor stage and differentiation status dependent.

Our data here indicate that cancer cells with reduced levels of endogenous PHLPP are more sensitive to re-introduction of PHLPP into the cells, and only a modest overexpression of PHLPP is enough to induce dephosphorylation of Akt effectively. On the other hand, if the level of endogenous PHLPP is already reduced basally, we found that further reduction of PHLPP expression by RNAi has limited effect on cell proliferation. In addition, knockdown of both PHLPP isoforms together by transiently transfecting cells with siRNAs did not enhance the effect of knocking down any single PHLPP isoform in DLD1 cells (data not shown). This may be due to the negative feedback control of the Akt pathway through p70S6K (Manning & Cantley, 2007; Manning et al., 2005), therefore, any hyperactivation of Akt beyond a certain threshold (as a result of knocking down both PHLPP isoforms) is prevented upon activation of the negative feedback loop (Brognard et al., 2007). Moreover, re-expressing PHLPP significantly increases the sensitivity of colon cancer cells to PI3K inhibition. As more selective PI3K inhibitors are being developed clinically for cancer treatment, determining the expression level of PHLPP in cancer may help to predict which patients will respond to PI3K inhibition.

In determining the molecular mechanism underlying PHLPP-mediated inhibitory effect in colon cancer cells, our results reveal that PHLPP overexpression leads to dephosphorylation of p27 at the site required for nuclear localization (Liang & Slingerland, 2003) and upregulation of p27 protein. However, the expression level of other cell cycle related proteins such as cyclins is not affected by PHLPP overexpression (Supplemental Fig. 2). p27 is a well characterized negative regulator of the cell cycle and loss of p27 expression is strongly associated with colorectal cancer progression (Hershko & Shapira, 2006). Previous studies have linked the loss TSC2 function to p27 downregulation (Rosner et al., 2006), thus, this increase of p27 expression observed in PHLPP overexpressing cells is likely the result of re-activation of TSC2. Interestingly, different PHLPP isoforms affects different phases of the cell cycle. It has been shown that accumulation of p27 in Skp2$^{-/-}$ mice results in cell cycle defects at both the G1-S and G2-M transitions (Nakayama et al., 2004). The distinct localization pattern of each PHLPP, as observed in normal epithelial cells (Fig. 1), may account for this differential effect. Given the existence of abundant phospho-proteins in cells and the limited numbers of protein phosphatases available, each PHLPP isoform is likely to have other yet to be identified substrates that also contribute to the final functional outcome.

In this study, we demonstrate that overexpression of either PHLPP isoform inhibits tumor formation in nude mice largely by reducing the rate of cell proliferation, since expression of PHLPP alone is insufficient to induce cell death (data not shown). However, previous

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studies have shown that overexpression of PHLPP promotes apoptosis in H157 and breast cancer cells (Brognard et al., 2007; Gao et al., 2005). This differential effect of PHLPP in different cancer cells may depend on the genetic background unique to each cell type. For example, the colon cancer cells used in this study are known to have activated Wnt pathway. The expression of Akt isoforms in different cancer cells may also play a role in determining the functional outcome of PHLPP. Unlike H157 cells, the predominant Akt isoform expressed in colon cancer cells is Akt1, while only a very small amount of Akt2 (less than 5% of Akt1) and no Akt3 are detected (Supplemental Fig. 3a; Samuels et al., 2005). Akt1 was dephosphorylated in PHLPP expressing cells whereas the phosphorylation of Akt2 was below the detection limit (Supplemental Fig. 3b). Future studies are needed to determine how PHLPP utilizes different signaling components downstream of Akt and interplays with other signaling pathways in different cancers. In summary, our current study identified both PHLPP isoforms as tumor suppressors in human colorectal cancer. The findings here provide a novel mechanism by which the tumorigenesis process of colon cancer is inhibited by PHLPP-mediated termination of oncogenic signals directly.

Materials and Methods

Antibodies

The anti-PHLPP1 and anti-PHLPP2 antibodies were obtained from Bethyl Laboratory (Montgomery, TX, USA). The following antibodies were from Cell Signaling (Danvers, MA, USA): rabbit antibodies against: Akt (detect total Akt protein), phospho-Akt (P473 for the Ser473 site and P308 for the Thr308 site), phospho-GSK3α/β (Ser21/Ser9), and phospho-Tuberin/TSC2 (Thr1462). Rabbit antibodies against PKC βII and PKC ζ were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-γ tubulin antibody was from Sigma-Aldrich (St Louis, MO, USA). Rabbit antibodies against phospho-p27 (T157 site) and total p27 were from R&D Systems (Minneapolis, MN, USA).

Immunohistochemical staining

Paraffin embedded normal and colorectal cancer tissue section slides were obtained from the tissue bank of Sealy Center for Cancer Cell Biology at UTMB and US Biomax (Rockville, MD, USA). The tissue sections were deparaffinized, rehydrated, and treated with hydrogen peroxide. Antigen retrieval was performed using Dako Target Retrieval Solution (DakoCytomation, Carpinteria, CA, USA) in a steamer. The sections were first blocked with avidin, biotin, and 5% normal goat serum and then incubated in a humidified chamber at 4 °C overnight with antibodies against PHLPP1 (1:500), PHLPP2 (1:500), P473 (1:50) or Ki67 (1:250). The samples were then incubated with biotin-labeled goat anti-rabbit secondary antibody and subsequently with HRP-avidin complex (Vector Laboratories, Burlingame, CA, USA). The staining was visualized using 3-amino-9-ethylcarbazol (AEC) substrate solution, and the sections were then counterstained with hematoxylin. The stained sections were visualized using a Nikon DXM1200 microscope. Scoring of PHLPP staining was done blindly according to the intensity of staining with a four-tier system (levels 0-3) (Reiner et al., 1990).

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Generation of PHLPP overexpressing stable cell lines

The colon cancer cell lines HCT116 and HT29 were cultured in McCoy's 5A media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin. The cells were transfected with HA-tagged PHLPP in the pcDNA/4-TO vector or the empty vector and subsequently selected with Zeocin (Invitrogen, 200 μg/ml). The resulting PHLPP overexpressing stable cells were termed HCT-P1, HCT-P2, HT29-P1, and HT29-P2, and the control cell lines HCT-Con and HT29-Con.

Lentivirus-mediated delivery of shRNA

The shRNAs for human PHLPP1 and PHLPP2 genes used in this study were constructed in pLKO.1-puro vector and purchased from Sigma-Aldrich (Stewart et al., 2003). Two constructs for each gene were chosen for this study, and the targeting sequences are as the following: for PHLPP1, #94: CCTGATAGTATCATCTGTGAA, and #97: CGAGGTCTTTCCCGAAGTTAT; and for PHLPP2, #59: CCTCTTCAGATCGTTTATGAT, and #60: GCCTGAACTTGTCCCATAATA. A plasmid carrying a non-targeting sequence was used to create the control cells. For virus packaging, the control or PHLPP-specific shRNA constructs were co-transfected with Mission lentiviral packing mix (Sigma-Aldrich) into 293T cells using FuGene 6. The virus containing media were collected, filtered, and overlaid onto DLD1 cells in the presence of polybrene (8 μg/ml) for 24 hours. The infected cells were then subjected to selection with puromycin (4 μg/ml).

Cell proliferation assay

To determine the rate of proliferation, equal numbers of cells were seeded into 12-well plates (2×10^4 cells/well). The numbers of cells were counted daily for 4 days using a cell counter (Beckman-Coulter, Fullerton, CA, USA). For the rescue experiments and the experiments including the PI3K inhibitor, the cells were seeded into 12-well plates and allowed to grow for an additional 72 hours. At the end of the experiments, the cells were fixed and stained with 0.5% crystal violet in 20% methanol for 30 minutes. After washing with water, the stained cells were dissolved in 1% SDS and absorbance at 570 nm was determined.

Cell cycle analysis

The cells grown in the regular growth or the serum-free media for 24-36 hours were collected, fixed in methanol, and stained with propidium iodide (25 μg/ml) as described previously (Gao et al., 2005). The cells were then subjected to the flow cytometry analysis. Quantification of the percentage cells in each cell cycle was obtained with Becton Dickinson FACSort (Franklin Lakes, NJ, USA).

Western Blotting

The cells were collected and resuspended in lysis buffer (50 mM Na_2HPO_4, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM DTT, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, 1 mM PMSF). The detergent-solublized cell lysate was obtained by centrifuging in a microcentrifuge at 16,000 g for 5 minutes. The ECL

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signals were detected and quantified using a FluoChem digital imaging system (Alpha Innotech, San Leandro, CA, USA).

Tumorigenesis analysis in xenografted nude mice

The stable HCT116 cells were collected in PBS and inoculated subcutaneously into 6-week-old female BALB/c-*nu/nu* mice at 1×10^6 cells/injection site. The tumor size was measured every 3-5 days with a caliper, and the tumor volumes was defined as (longest diameter) × (shortest diameter)^2/2. At the end of experiments, the mice were sacrificed and the tumors dissected from the individual mouse were fixed in 10% buffered formalin. The paraffin embedded samples were prepared, and 5 μm sections were used for IHC staining. All animal procedures were done in the nude mouse facility using protocols approved by the UTMB Animal Care and Use Committee.

Statistical Analysis

The results from the experiments to assess the rate of proliferation, G1/S ratio, numbers of cells in G2, and numbers of Ki-67 positive cells were summarized using mean ± SEM and pairwise comparisons were carried out using two-sample t-tests. IHC intensity staining scores (0-3) of PHLPP1 and PHLPP2 in patient specimens were dichotomized into low (0-1) or high (2-3) expression in normal and tumor tissues. Comparisons were performed using the Wilcoxon rank-sum test for intensity scores or the chi-square test for dichotomized PHLPP IHC expression levels. Spearmans coefficient was calculated to assess the correlation in intensity scores between the two PHLPP isoforms. Tumor growth in nude mice studies were summarized at each time point of follow-up and analysis was performed using longitudinal models to account for repeatedly-measured tumor volume over time within each mouse.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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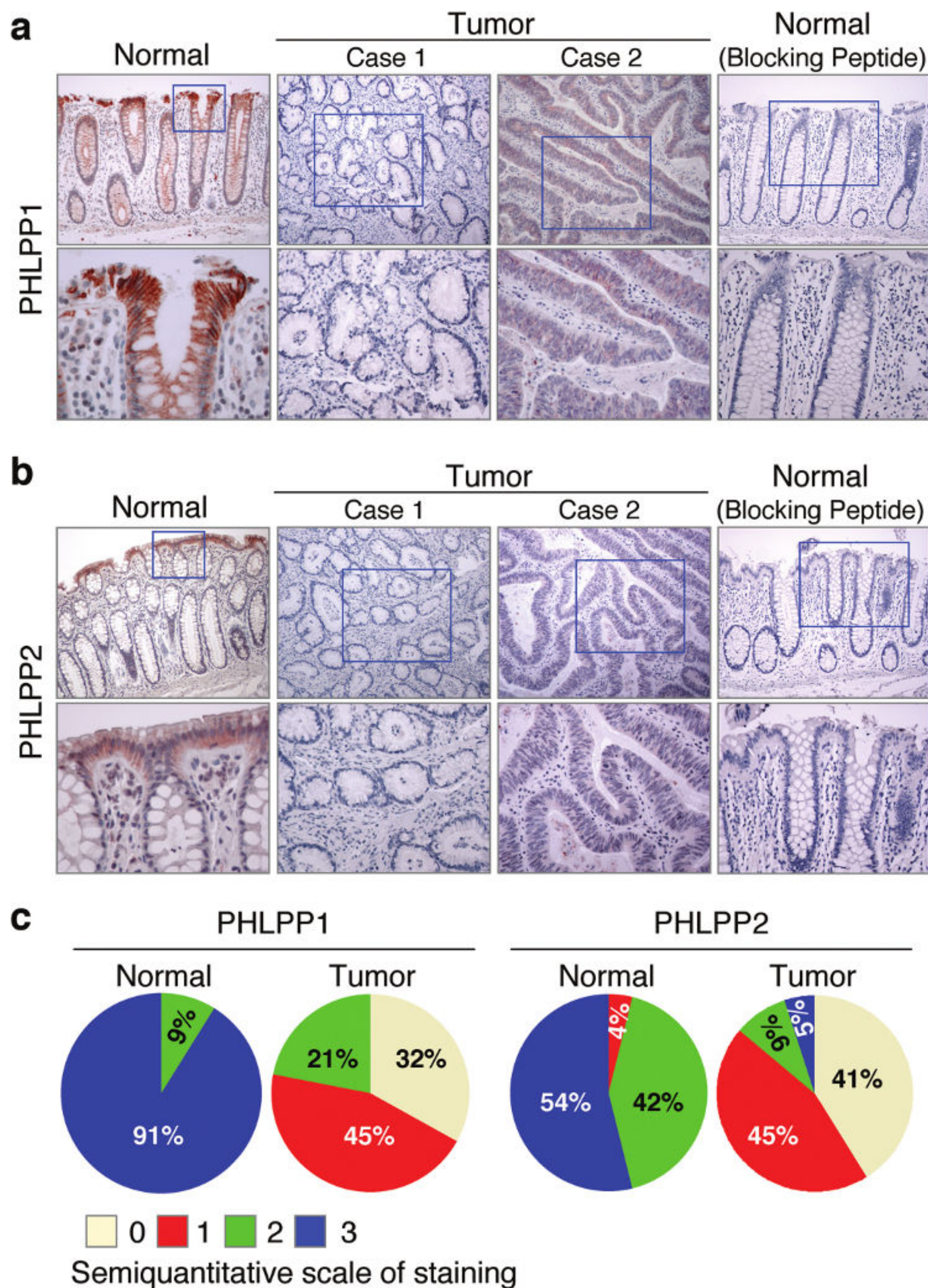

Figure. 1.

Loss of PHLPP expression in colorectal cancer clinical specimens. Colorectal cancers with proximal, transverse, or distal tumors and adjacent normal mucosa were analyzed for expression of PHLPP1 and PHLPP2 isoforms using IHC staining. (**a**) and (**b**) representative images taken from the normal and tumor specimens stained with the PHLPP isoform specific antibodies. Images shown in the upper panels were obtained with a 10× objective, and the enlarged images of areas enclosed by the blue boxes are shown in the panels directly below. The images in the right most panels were taken from the normal specimens stained

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with the PHLPP antibodies that were pre-absorbed with the peptide antigens. (**c**) Quantitative representation of summarized results obtained from the IHC staining experiments. The intensity of staining for each protein was scored with a four-tier system (0-3) (Reiner et al., 1990). A total of 16 sets of pair-matched samples (from 16 patients) are included. Additional samples screened are in the format of tissue arrays that contain tumor tissue sections from 40 colon cancer patients and 8 normal colon tissue sections. Thus, the total numbers of normal samples screened are 24 and the tumor samples are 55 for PHLPP1 and 56 for PHLPP2. The pie charts indicate the percentage of samples in each tier category.

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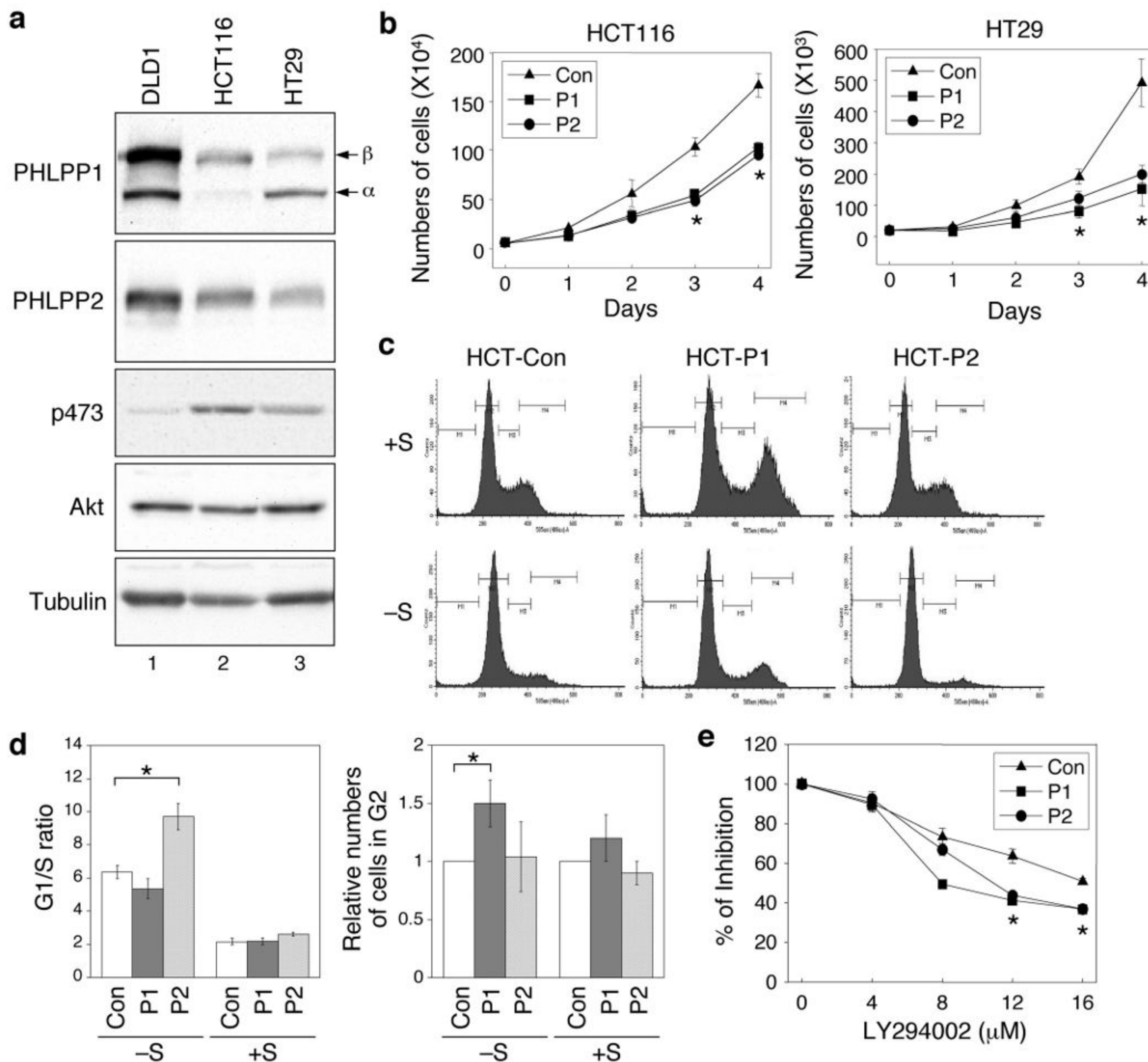

Figure 2.

Overexpression of PHLPP inhibits cell proliferation. (**a**) Expression of PHLPP1 and PHLPP2 in colon cancer cell lines. Cell lysates prepared from DLD1, HCT116, and HT29 cells (lanes 1-3, respectively) were analyzed using Western blotting. The expression of endogenous PHLPP1 and PHLPP2 was detected using the anti-PHLPP1 and anti-PHLPP2 antibodies. The phosphorylation and total protein expression of Akt were detected using the P473 and anti-Akt antibodies, respectively. Tubulin was detected using the anti-γ tubulin mAb. (**b**) Graphs showing the rate of proliferation in PHLPP overexpressing cells. Equal numbers of cells were seeded at day 0 into regular growth media. The cells were allowed to grow for 4 days, and the cell numbers were examined daily using a cell counter. The results obtained from HCT-Con, HCT-P1, and HCT-P2 cells are shown in the left graph, while the

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results of HT29-Con, HT29-P1, and HT29-P2 cells are shown in the right graph. Data represent the mean ± SEM (n=3). (**c**) HCT-Con, HCT-P1, and HCT-P2 cells grown in the regular growth or serum-free media were analyzed using flow cytometry. Graphs shown are the representative results of cell cycle analysis. (**d**) Graphs showing quantified results of the experiments shown in (**c**). The G1/S ratio was calculated based on the percentage of cells in G1 and S phase of the cell cycle. Data represent the mean ± SEM (n=3). (**e**) Overexpression of either PHLPP isoform sensitized HCT116 cells to LY294002 induced growth inhibition. HCT-Con, HCT-P1, and HCT-P2 cells were kept in regular growth media supplemented with 0, 4, 8, 12, and 16 μM of LY294002 for 48 hours. The cells were then fixed and stained with crystal violet, and the absorbance at 570 nm was measure. The absorbance obtained from HCT-Con cells in media without LY294002 was treated as the control point (i.e. 100% cell growth with no inhibition), and the percentage of inhibition was calculated by normalizing other OD measurements to the control point. The graph represents the mean ± SEM (n=3). The asterisks in all graphs indicate $p<0.05$ as determined by two-sample t-tests.

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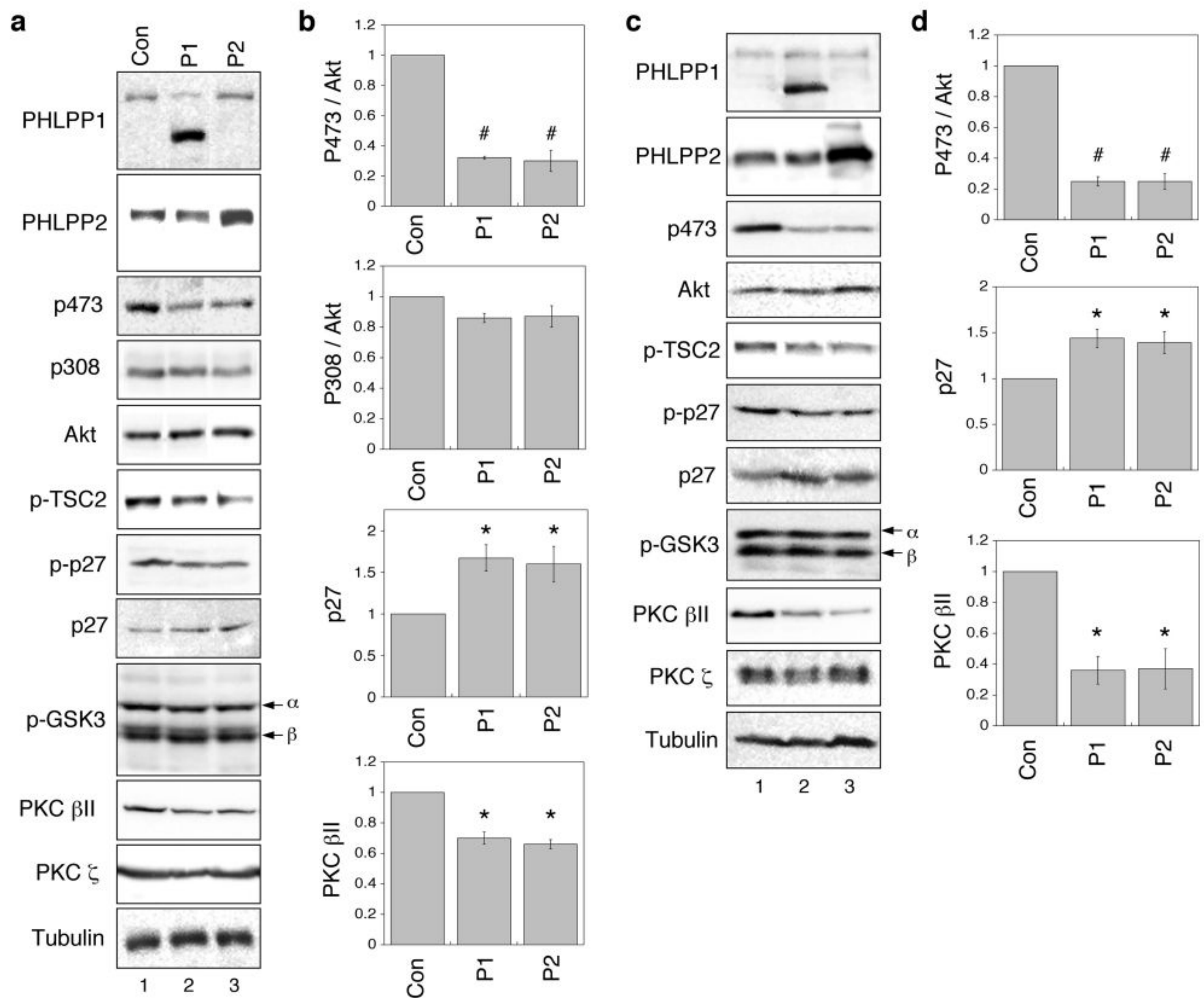

Figure 3.

Overexpression of PHLPP negatively regulates both Akt and PKC βII in colon cancer cells. Western blots of cell lysates prepared from the PHLPP overexpressing stable cells were analyzed using the following antibodies to show the phosphorylation status or the total protein expression: PHLPP1, PHLPP2, P473, P308, Akt, phospho-TSC2 (T1462 site, p-TSC2), phosph-p27 (T157 site, p-p27), p27, phospho-GSK3 (S21/S9 sites, p-GSK3), PKC βII, PKC ζ, and γ tubulin antibodies. (**a**) PHLPP-mediated regulation of Akt, Akt substrates, and PKC in HCT-Con, HCT-P1, and HCT-P2 cells (lanes 1-3, respectively). (**b**) Graphs showing quantified data from three independent experiments as shown in (**a**). (**c**) PHLPP-mediated regulation of Akt, Akt substrates, and PKC in HT29-Con, HT29-P1, and HT29-P2 cells (lanes 1-3, respectively). (**d**) Graphs showing quantified data from three independent experiments as shown in (**c**). The ECL signals were captured and quantified using a FluorChem digital imager (Alpha Innotech). For all graphs shown, relative phosphorylation of Akt at Ser473 (P473/Akt) or T308 (P308/Akt) was determined by normalizing ECL signals of the P473 or P308 antibodies to those of the Akt antibody, and the relative p27 and PKC βII expression was calculated by normalizing ECL signals of the p27 or PKC βII

antibodies to those of tubulin. The control cells (Con) were set to 1, and two-sample t-tests were performed to compare the difference between the PHLPP overexpressing cells and the control cells. Data represent mean ± SEM (n=3; # $p<0.001$ and * $p<0.05$).

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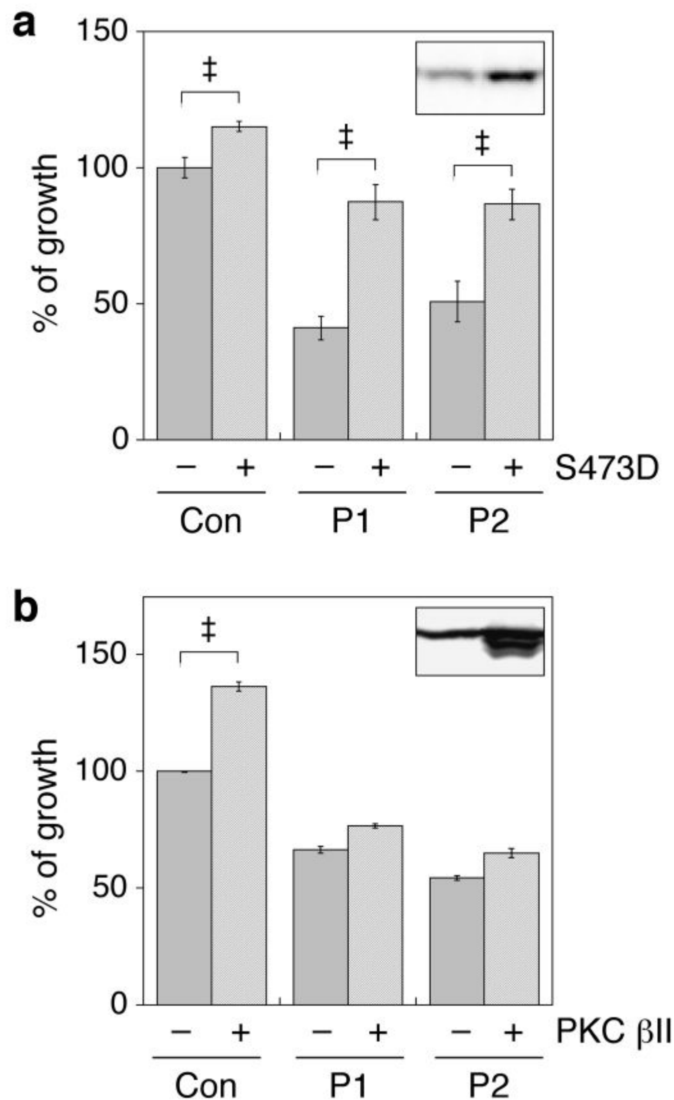

Figure 4.

Overexpression of an active form of Akt but not PKC βII alleviates PHLPP-mediated growth inhibition. HCT-Con, HCT-P1, and HCT-P2 stable cells were transiently transfected with vector or Akt mutant S473D in (**a**); and transfected with vector or PKC βII in (**b**). For all data points, equal numbers of transfected cells were reseeded approximately 16 hours post transfection and allowed to grow for 72 hours. The cells were then fixed and stained with crystal violet, washed, and the absorbance at 570 nm was measure. The two-sample t-tests were performed to compare the difference between the vector and Akt/S473D or PKC βII transfected cells. Data represent mean ± SEM (n=3; ‡ p<0.01). The blot insert in the graph (**a**) shows the expression of Akt, and the blot insert in the graph (**b**) shows the expression of PKC βII in the transfected cells. Note that since the cellular PKC activity is determined by the total PKC expression rather than the phosphorylation status, overexpression of wild-type PKC βII is sufficient to increase the amount of kinase available in cells. The expression construct used here encodes a rat PKC βII protein which has a slightly smaller molecular weight than the endogenous human homologue.

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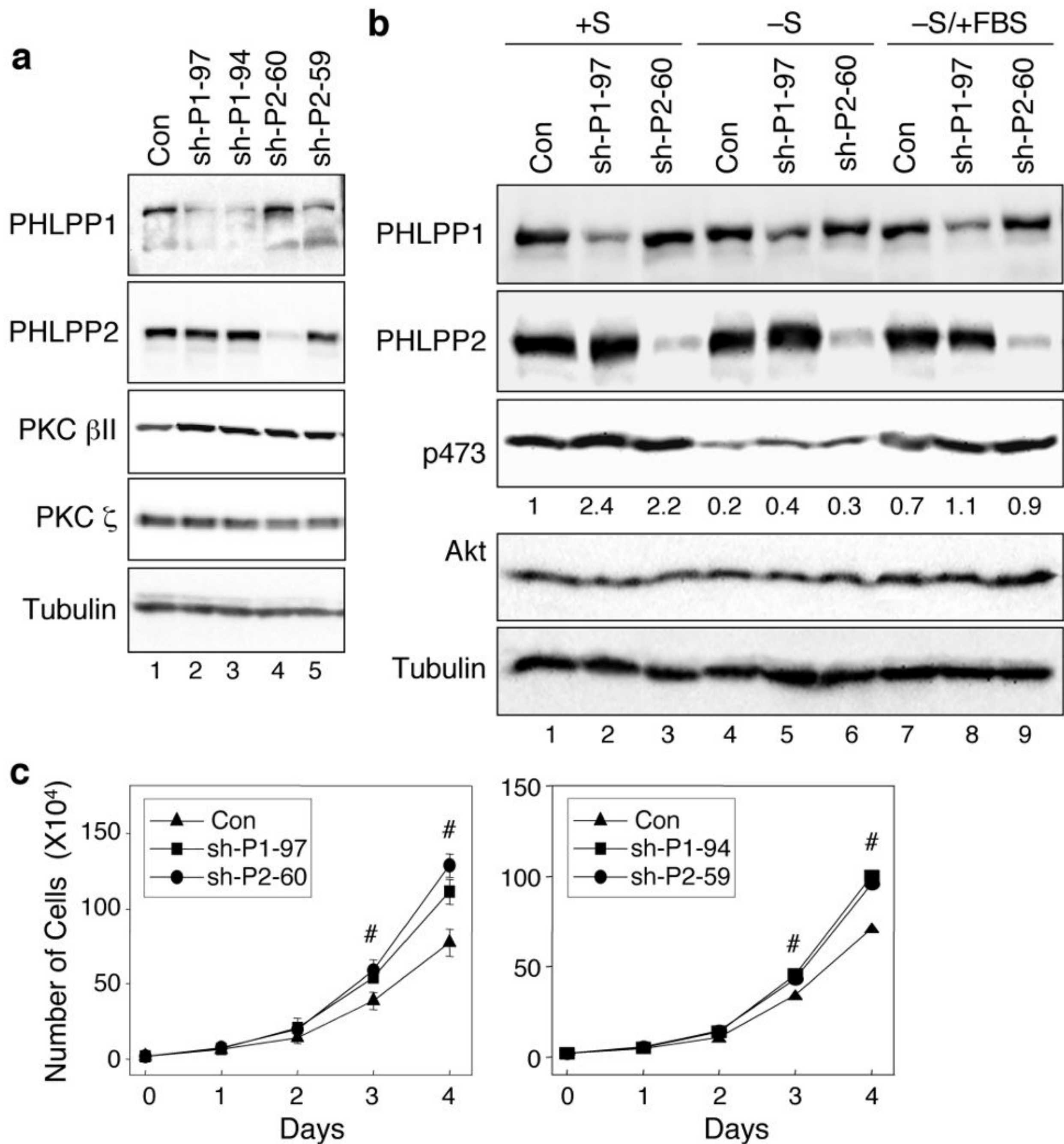

Figure 5.

Knockdown of PHLPP isoforms in DLD1 cells promotes proliferation. (**a**) Knockdown of PHLPP expression in DLD1 cells resulted in an upregulation of PKC βII. Stable knockdown cells were created to deplete expression of PHLPP1 or PHLPP2 in DLD1 cells. Cell lysates prepared from Con, sh-P1-97, sh-P1-94, sh-P2-60, and sh-P2-57 cells (lanes 1-5, respectively) were analyzed using Western blotting. The following antibodies were used: PHLPP1, PHLPP2, PKC βII, PKC ζ, and γ tubulin antibodies. (**b**) DLD1 stable knockdown cells including Con, sh-P1-97, and sh-P2-60 cells were either kept overnight in either serum

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containing media (+S, lanes 1-3), serum-free media (–S, lanes 4-6), or serum-free media and then switched back to serum containing media for 30 minutes (–S/+FBS, lanes 7-9). The cell lysates were prepared and analyzed for Akt phosphorylation. The expression of endogenous PHLPP was detected using the PHLPP1 and PHLPP2 antibodies. The phosphorylation of endogenous Akt was detected using the P473 antibody, while the total Akt was detected using the Akt antibody. Tubulin was probed with the γ tubulin antibody. The relative phosphorylation of Akt was obtained by normalizing ECL signals of P473 to those of total Akt and is indicated by the numbers below the P473 panel. The phosphorylation level of Akt in the control cells in serum was set to 1, and all other conditions were normalized accordingly. (**c**) Graphs showing the rate of proliferation in PHLPP knockdown cells. Two sets of experiments were performed to compare sh-P1-97 and sh-P2-60 or sh-P1-94 and sh-P2-59 to the control cells separately. Equal numbers of stable knockdown cells were seeded at day 0 in the regular growth medium. The cells were allowed to grow for additional 4 days, and the cell numbers were counted daily using a Coulter cell counter. For statistical analysis, the data obtained from the knockdown cells were compared to the control cells and the p-values were determined by two-sample t-tests. Data represent the mean ± SEM (n=3, # $p<0.001$).

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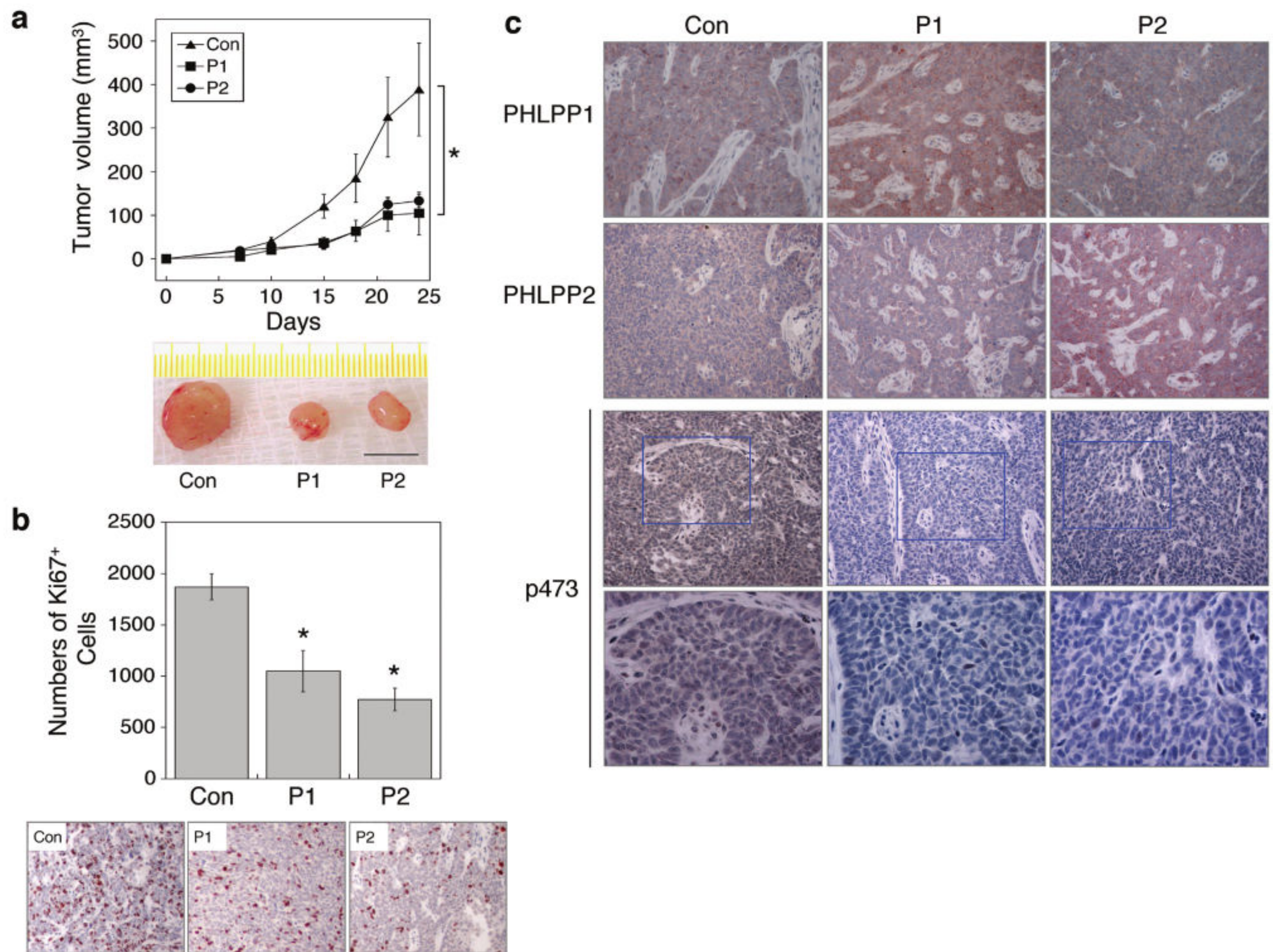

Figure 6.

Overexpression of PHLPP isoforms reduces the tumorigenic potential of colon cancer cells *in vivo*. (**a**) Nude mice of BALB/c background were inoculated subcutaneously with HCT-Con, HCT-P1, and HCT-P2 cells. The size of the tumors was measured every 3-5 days. Four mice were used in each group, and the cells were inoculated at two sites in each mouse. Thus, total 8 tumors were measured and averaged in each group. Data in the graph represent the mean ± SEM, and the analysis was based on a longitudinal model to account for repeated measurements with each mouse (the asterisk indicates $p<0.0001$ for both HCT-P1 and HCT-P2 compared to HCT-Con). Representative tumors isolated from each group are shown below the graph, and the grey bar in the picture represents 1cm. (**b**) The rate of cell proliferation was decreased in tumors derived from PHLPP expressing cells. The tumors isolated from mice injected with HCT-Con, HCT-P1, or HCT-P2 cells were fixed, sectioned, and stained with the anti-Ki67 antibody. The Ki67 positive cells were viewed and counted under a microscope using the 10× objective. The numbers from 4 random viewing fields were averaged, and representative images are shown. Data in the graph represent the mean ± SEM (n=4, * $p<0.05$). (**c**) The phosphorylation status of Akt in tumors derived from HCT116 stable cells. The tissue sections from the tumors formed by HCT-Con, HCT-P1, or HCT-P2 cells were stained with the PHLPP1 (upper panels), PHLPP2 (middle panels), or

P473 (lower panels) antibodies. For P473 stained sections, the areas enclosed by the blue boxes in the top panels were enlarged and shown directly below.

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Table 1

Descriptive statistics of PHLPP1 and PHLPP2 using IHC staining score

	Median (Range)	Mean ± SD
PHLPP1		
Normal Tissue (n=24)	3.0 (2.0 – 3.0)	2.92 ± 0.28
Tumor Tissue (n=55)	1.0 (0 – 2.0)	0.89 ± 0.73
PHLPP2		
Normal Tissue (n=24)	3.0 (1.0 – 3.0)	2.50 ± 0.59
Tumor Tissue (n=56)	1.0 (0 – 3.0)	0.78 ± 0.82

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Table 2

Spearman Correlation Between PHLPP1 and PHLPP2 Using Staining Score

	Spearman Correlation	**p-value**
Normal Tissue		
PHLPP1 vs. PHLPP2 (n=24)	0.30	0.16
Tumor Tissue		
PHLPP1 vs. PHLPP2 (n=56)	0.75	<0.0001

Note: There was a statistically significant positive correlation between PHLPP1 and PHLPP2 in the tumor tissue ($r = 0.75$, $p < 0.0001$) while this correlation between the two proteins did not hold true for normal tissue ($r = 0.30$, $p=0.16$).