HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2019 April 05.

Published in final edited form as:

Oncogene. 2018 April ; 37(17): 2225–2236. doi:10.1038/s41388-017-0061-7.

PHLPP1 mediates melanoma metastasis suppression through repressing AKT2 activation

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Abstract

PI3K/AKT pathway activation is thought to be a driving force in metastatic melanomas. Members of the pleckstrin homology (PH) domain leucine-rich repeat protein Ser/Thr specific phosphatase family (PHLPP1 and PHLPP2) can regulate AKT activation. By dephosphorylating specific serine residues in the hydrophobic motif, PHLPP1and PHLPP2 restrain AKT signalings, thereby regulating cell proliferation and survival. We here show that PHLPP1 expression was significantly downregulated or lost and correlated with metastatic potential in melanoma. Forcing expression of either PHLPP1 or PHLPP2 in melanoma cells inhibited cell proliferation, migration and colony formation in soft agar; but PHLPP1 had the most profound inhibitory effect on metastasis. Moreover, expression of PH mutant forms of PHLPP1 continued to inhibit metastasis, whereas a phosphatase-dead C-terminal mutant did not. The introduction of activated PHLPP1-specific targets AKT2 or AKT3 also promoted melanoma metastasis, while the non-PHLPP1 target AKT1 did not. AKT2 and AKT3 could even rescue the PHLPP1-mediated inhibition of metastasis. An AKT inhibitor blocked the activity of AKT2 and inhibited AKT2-mediated tumor growth and metastasis in a preclinical mouse model. Our data demonstrate that PHLPP1 functions as a metastasis suppressor through its phosphatase activity, and suggest that PHLPP1 represents a novel diagnostic and therapeutic marker for metastatic melanoma.

Keywords

PHLPP1; AKT1; AKT2; AKT3; metastasis suppressor; melanoma

Introduction

Protein kinases and phosphatases maintain a balance between protein phosphorylation and dephosphorylation, allowing them to control a broad range of physiological processes

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Conflict of interest

The authors have no conflict of interest to declare.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

including cell proliferation, differentiation, motility, migration, and survival. Dysregulation of one or both leads to pathophysiological diseases.[1, 2] Hyperactivation of protein kinases is particularly prevalent in cancers.[3–5] For instance, protein kinase B (PKB), also known as AKT, a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation and cell migration, is commonly activated in numerous types of tumors.[6, 7] In melanoma, 60-70% of tumors exhibit activated AKT.[8, 9] Moreover, disease progression correlates with progressively activated AKT; for example, activated AKT was found in 17%, 43%, 49% and 77% of 292 lesions of normal nevi, dysplastic nevi, primary melanoma and metastatic melanomas, respectively.[10] These data suggest that AKT activity plays a significant role in melanoma progression to metastatic status.

The well-studied intracellular activator of AKT is phosphoinositide 3-kinase (PI3K).[11] Growth factors and cytokines bind to cell surface receptors to trigger PI3K activation. Activated PI3K phosphorylates phosphatidylinositols generating the lipid PI (3,4,5) P3. PI (3,4,5) P3 then binds to the PH domains of the AKT family and phosphoinositide-dependent kinase (PDK1), recruiting them to the cell membrane.[12] PDK1 then phosphorylates the AKT activation loop (Thr308). After phosphorylation of their hydrophobic motif (Ser473) by PDK2 (also known as mTorc2), AKT becomes fully activated and targets an array of substrates to regulate cell survival, proliferation, metabolism and motility.[7, 12, 13]

The AKT family consists of three main members: AKT1, AKT2, and AKT3.[7, 12, 13] They share over 80% homology and comprise three conserved functional structures of a pleckstrin homology (PH) domain at N-terminal, a kinase domain in central and a hydrophobic domain in C-terminal.[7, 12] Although their domain architecture and upstream regulation are similar, studies have shown that their expression and biological functions are not redundant. AKT1 is expressed ubiquitously in all tissue and controls cell proliferation and apoptosis. AKT2 is highly expressed in insulin-responsive tissues such as liver and muscle and maintains glucose homeostasis. The AKT3 expression is more specific to neuronal tissue and regulates brain development.[14, 15] More recently, AKT3 has been implicated in melanomagenesis.[9] AKT1 has been found to promote tumor growth and suppress metastasis in breast cancers, [14–18] while its activation might promote the melanoma metastases in a mouse model.[19] In contrast, AKT2 promotes metastasis in breast and ovarian cancers [16, 20] as well as colorectal cancer.[21] These data suggest that different AKT isoforms control various cellular processes and pathophysiological processes such as metastasis, supporting the hypothesis that inhibition of specific AKT isoforms is a more viable therapeutic strategy for metastatic disease.

The best-known intracellular inhibitor of activated AKTs is the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN).[22] PTEN dephosphorylates the plasma member lipid phosphatidylinositol-3, 4, 5-trisphosphate (PIP3) and enables it to antagonize the PI3K upstream of AKT effectively, indirectly results in the inactivation of AKT, thereby inhibiting cell proliferation and promoting apoptosis [23, 24] as well as cell migration.[25] However, there are many examples of cancer cells with elevated AKT phosphorylation that have intact PTEN expression.[26, 27] Recent studies reported that AKT activation can be directly repressed by members of the pleckstrin homology (PH)

domain leucine-rich repeat protein Ser/Thr specific phosphatase family (PHLPP1 and PHLPP2), which catalyzes the dephosphorylation of the AKT hydrophobic phosphorylation motif (serine resident).[27, 28] Specifically, PHLPP1 can dephosphorylate serine 474 of AKT2 and serine 472 of AKT3, whereas PHLPP2 phosphatases serine 473 of AKT1 and serine 472 of AKT3.[27, 28] By dephosphorylating specific serine residues within the hydrophobic motif, PHLPP1, and PHLPP2 control the degree of agonist-evoked signaling by AKT proteins, thereby regulating cell proliferation and survival.[27, 28] Together, the findings from these studies led us to explore the function of PHLPPs and the role of AKT isoforms in regulation of melanoma metastasis.

We here find that PHLPP1 expression is significantly downregulated or lost and correlated with metastatic potential in melanoma. Forcing expression of PHLPP1 inhibited metastasis in melanoma cells, whereas knockdown of PHLPP1 in human melanoma cells enhanced metastatic potential. Unexpectedly, PHLPP2 had little influence on metastatic ability *in vivo*, despite the fact that both PHLPP1 and PHLPP2 were able to inhibit *in vitro* cell proliferation and migration as well as colony formation in soft agar. Notably, the introduction of PH mutant forms of PHLPP1 still blunted the metastatic potential of both cell lines, whereas a phosphatase-deficient C-terminal mutant did not affect metastasis. Moreover, overexpression of either AKT2 or AKT3 promoted melanoma metastasis, while knockdown and or knockout of AKT2 or AKT3 blocked metastasis. Transfection of the activated form of AKT2 or AKT3 rescued PHLPP1-mediated inhibition of metastasis. We also show that PHLPP1 negatively regulates the activation of AKT2/3, thereby inhibiting the metastatic potential of melanoma cells. Our data indicate for the first time that PHLPP1 plays a critical role in inhibiting metastasis through its phosphatase activity repressing AKT2/3 and suggest that PHLPP1 is a novel diagnostic marker and therapeutic target for metastatic melanoma.

Results

PHLPP1 suppresses metastasis in human melanoma.

To explore the role of PHLPPs in melanoma, we examined the expression of the PHLPP family in a panel of highly and poorly metastatic melanoma cell lines by western blot. We found that PHLPP1 was commonly lost or reduced in melanoma cell lines (71.4%, 15 out of 21) compared with melanocytes. In contrast, with PHLPP2 there was no consistent reduction, or increase, in melanoma cell lines compared with melanocytes. Notably, highly metastatic cell lines significantly lost PHLPP1 expression (69.2%, 9 out of 13) compared with poorly metastatic cell lines (0 out of 8) (Figure 1A). We also observed that levels of PHLPP1 transcripts were significantly reduced in metastatic melanoma samples compared to primary melanomas (Figure 1B), whereas PHLPP2 transcripts were unchanged in metastatic melanomas (GSE7553 data set) (Figure 1C). This raises the possibility that PHLPP1 may be involved in the regulation of metastasis in melanoma. To assess a possible role for PHLPP family in metastasis, we introduced PHLPP1 and PHLPP2 into highly metastatic human melanoma A375sm cells and tested the metastatic potential of stably transfected cells *in vivo*. Forced expression of either PHLPP1 or PHLPP2 decreased activation of AKT (pan-phosph-AKT ser473) (Figure 1D). We found that expression of either PHLPP protein member was able to inhibit pulmonary metastasis of A375sm cells compared to empty

vector control; however, only PHLPP1's effect was profound with statistic significant in both tail-vein transplantation assay in SCID mice (p=0.003, Figure S1A) and orthotopic footpad transplantation assay in NSG mice (p=0.0027, Figure 1E). To further confirm the function of PHLPPs on metastasis, we knocked down endogenous PHLPP1 and PHLPP2 by shRNAs in poorly metastatic human melanoma A375p cells (Figure 1F). When introduced into NSG mice by footpad transplantation, knockdown of PHLPP1 in A375p cells significantly enhanced pulmonary metastasis in all transfected cells when compared with the empty vector control ($p<0.05$), whereas blocking PHLPP2 only slightly increased metastasis with no statistically significant difference ($p>0.05$), (Figure 1G). These data suggest that the PHLPP family plays a role in regulating metastasis, with PHLPP1 being the dominant member.

PHLPP1 inhibits melanoma metastasis through its phosphatase activity.

The PHLPPs were recently identified as Ser/Thr-specific phosphatases belonging to the pleckstrin homology (PH) domain leucine-rich repeat protein phosphatase family.[27, 28] To determine whether PHLPP1 phosphatase activity is required for suppressing metastasis, we introduced the wildtype (WT), C-terminal deleted mutant (ΔC) or PH domain deleted mutant (ΔPH) of PHLPP1- and PHLPP2-expressing plasmids into human A375sm cells (Figure 2A). Two cell clones of each stably transfected cell were selected for testing the functions of PHLPP1 *in vitro* and *in vivo*. Overexpression of either the PHLPP1 WT or the PHLPP1 ΔPH mutant significantly inhibited A375sm cell growth, motility, invasiveness (Figure 2B-D). Moreover, both WT and ΔPH forms of PHLPP1 inhibited colony formation in soft agar in vitro (Figure 2E, F) and pulmonary metastasis using tail-vein transplantation assay in SCID mice (Figure 2G) and orthotopic footpad transplantation assay in NSG mice (Figure S1B), the degrees to statistic significant are based on the expression level (Figure 2A, F-G). However, expression of the phosphatase-dead PHLPP1 ΔC mutant did not alter *in vitro* cell growth, motility, invasiveness or growth in soft agar, and did not affect metastasis *in vivo* (Figure 2). Additionally, while expression of PHLPP2 could significantly inhibit cell growth, invasion, and colony formation in soft agar, it but did not affect cell motility *in vitro* or metastasis *in vivo* (Figure 2). These data strongly suggest that PHLPP1 inhibits melanoma metastasis through its phosphatase activity.

PHLPP1 deactivates Akt2 and suppresses metastasis.

PHLPP1 and PHLPP2 have recently been found to control AKT's kinase signaling by catalyzing the dephosphorylation of the hydrophobic AKT phosphorylation motif (Ser/Thr).[27, 28] We, therefore, sought to determine if the PHLPPs regulate metastasis through the dephosphorylation of AKT family members. Western blot analysis was used to demonstrate that overexpression of both PHLPP1 and PHLPP2 decreased the phosphorylation of pan-AKT (Figure 1D); conversely, knockdown of both PHLPP1 and PHLPP2 increased the phosphorylation of pan-AKT (Figure 1F). Next, an enzyme-linked immunosorbent assay (ELISA) was used to determine which AKT(s) were targets of the PHLPPs phosphatase in melanoma cells. Specifically, we measured the effects of PHLPP1 and its mutant forms as well as PHLPP2 overexpression or knockdowns on the levels of activated AKT1 (pAKT1), AKT2 (pAKT2) and AKT3 (pAKT3). We found that overexpression of PHLPP2 specifically decreased pAKT1 and pAKT3 but not pAKT2 (Figure S2), while PHLPP2 shRNA

knockdown increased pAKT1 and pAKT3 but not pAKT2 (Figure S3). However, overexpression of PHLPP1 decreased pAKT2 and pAKT3 but not pAKT1 (Figure S2), whereas PHLPP1 shRNA knockdown increased pAKT2 and pAKT3 but not pAKT1 (Figure S3). Notably, the phosphatase-dead PHLPP1 ΔC mutant lost the capacity to inhibit the formation of pAKT2 and pAKT3, whereas the PHLPP1 ΔPH mutant still decreased levels of pAKT2 and pAKT3 (Figure S2).

These data indicate that PHLPP1 selectively dephosphorylates AKT2 and AKT3, which may influence its ability to regulate metastasis. To address this, we introduced constitutively activated AKT1, AKT2, and AKT3 into human A375p and mouse B16F1 cells and tested their metastatic potential. Activated AKT1, AKT2 and AKT3 in transfected A375p cells were significantly increased by 2- to 3-fold compared with empty vector control (Figure 3A-D). However, only AKT2 and AKT3 promoted pulmonary metastasis (Figure 3E). Similar to human A375p cells, mouse B16F1 cells with transfected activated AKT2 and AKT3 had significantly higher number of pulmonary metastasis compared to cells transfected with empty vector (Figure 3F-J). Surprisingly, activated AKT1 had no effect in human A375p melanoma cells (Figure 3E) and decreased the number of pulmonary metastasis in mouse B16F1 cells (Figure 3J).

These results suggest that activated AKT2 and AKT3 are involved in the regulation of metastasis in melanoma. To further confirm the function of AKT isoforms in metastasis, we knocked down endogenous AKT1, AKT2 or AKT3 in highly metastatic A375sm cells using specific AKTs shRNAs (Figure 3K) and found that knockdown of AKT2 and AKT3 blocked the metastatic potential significantly, but knockdown of AKT1 did not (Figure 3L). Notably, these findings were further confirmed using genome editing approach for specifically knockout of AKT2 and AKT3 in A375sm cells (Figure 3M, N). Our data demonstrate that AKT2 and AKT3 play a significant role in regulating metastasis in melanoma. Because PHLPP1 can block activation of AKT2 through dephosphorylation at Ser 474, and AKT3 at Ser 472, we hypothesized that expression of AKTs would abrogate the inhibition of PHLPP1 and restore metastatic potential. To study this, we introduced constitutively activated AKT1, AKT2, or AKT3 into human A375sm cells stably expressing PHLPP1, PHLPP1ΔC or PHLPP2, and determined their metastatic potential *in vivo*. Figure 4 shows that forced expression of AKT1, AKT2 or AKT3 caused a 2- to 4-fold significant increase in total AKT (T-Akt) (Figure 4A, B, and C) and phosphorylated AKT (p-Akt) (Figure 4D, E, and F), as well as activation of AKT-Thr-308 (Figure 4G); these changes did not affect the levels of phosphorylated Erk1/2 (Figure 4H) and total Erk1/2 (Figure 4I). However, forced expression of AKT2 significantly increased pulmonary metastasis not only in cells with PHLPP1 but also in cells with PHLPP1ΔC and PHLPP2; AKT3 increased metastasis in cells with PHLPP1and PHLPP1ΔC but not in cells with PHLPP2 (Figure 4J, K). In contrast, AKT1 expression did not affect metastasis in any cells (Figure 4J, K). These data demonstrate that AKT2, and to a lesser extent AKT3, can rescue the inhibition of PHLPP1-mediated metastasis and promote metastasis in melanoma, suggesting that PHLPP1 inhibits tumor metastasis by negatively regulating AKT2/3 activity.

Inhibition of AKT2 by MK2206 blocks tumor growth and metastasis *in vivo.*

To further confirm the importance of AKT2 in the regulation of metastasis in melanoma, we first treated human A375sm and A375p cells stably expressing activated AKT2 (A375p AKT2) with the AKT allosteric specific inhibitor MK2206 [29] *in vitro*. Using western blot analysis, we found that MK2206 inhibited AKT phosphorylation in a dose-dependent manner (Figure 5A). To determine which AKT isoforms would be affected by MK2206, ELISA assays were used to examine the activities of various AKT isoforms. We noted that while MK2206 could inhibit the activity of all three AKT isoforms to some extent (Figure 5B, C, D), AKT2 phosphorylation was most severely affected in both with and without transfected AKT2 cell lines in a similar way (Figure 5C). To further confirm the role of the AKTs in metastasis, we treated mice bearing A375p AKT2 melanoma cells with MK2206. Immediately following or 7 days after tail vein injection of A375p AKT2 cells into SCID mice, two different doses of MK2206 were administered to mice over the next 3- to 4-weeks. Mice treated with either dose or initiated time point bore a significantly lower number of pulmonary metastases relative to vehicle-treated control mice (Figure 5F). Moreover, treatment with MK2206 from seventeen days after subcutaneous A375sm cells transplantation inhibited tumor growth significantly (Figure 5G, H). Consistent with the results from treated cells *in vitro*, the tumors treated with MK2206 showed weaker AKT activation by immunohistochemical staining (Figure 5I, J). Tumors with MK2206 treatment exhibited a dramatic reduction of phosphorylation of AKT2 and AKT3, but not AKT1 (Figure 5K), as well as no change in Erk activity (Figure 5L). Together, our data demonstrate that inhibition of AKT2, and to a lesser degree AKT3, by MK2006 blocked melanoma growth and metastasis.

Discussion

PI3 kinase/AKT pathway activation is thought to be a driving force in metastatic melanoma. AKT kinase signaling can be regulated by members of the pleckstrin homology (PH) domain leucine-rich repeat protein Ser/Thr specific phosphatase family (PHLPP1 and PHLPP2), which catalyzes the dephosphorylation of the AKT hydrophobic phosphorylation motif (Ser/Thr).[27, 28] By dephosphorylating specific serine residues within their hydrophobic motifs, PHLPP1 and PHLPP2 restrain the AKT signalings, thereby regulating cell proliferation and survival.[27, 28] Although several recent studies have reported the role that PHLPPs plays in tumorigenesis in various tumor types,[27, 28, 30–32] the functional importance of PHLPP1 and PHLPP2 in tumor metastasis remains to be elucidated.

In this study, we show that PHLPP1 expression is significantly downregulated or lost, and correlates with metastatic potential in melanoma. We assessed the effects of PHLPP1 or PHLPP2 on melanoma metastasis and showed that PHLPP1 could inhibit the metastatic potential of melanoma through its phosphatase activity. We then examined the functions of the various AKT isoforms on melanoma metastasis and indicate that AKT2 and AKT3 promote the metastatic ability of melanoma cells. We further demonstrate that PHLPP1 suppresses melanoma metastasis through inhibition of AKT2, and to a lesser extent AKT3. Additionally, we show that the AKT inhibitor MK2206 can prevent the activation of AKT2 and block melanoma growth and metastasis in preclinical models. Our data, therefore,

indicate that PHLPP1 functions as a metastasis suppressor through its phosphatase activity, and suggest that PHLPP1 represents a novel diagnostic and therapeutic marker for metastatic melanoma.

The functional importance of PHLPPs in tumor progression has been reported in recent studies.[27, 28, 30–35] Inactivation of PHLPP1 and/or PHLLP2 has been found in various cancers such as colon,[30] breast, [27] prostate [31] and pancreatic [32] cancers, lymphoma,[33] glioblastomas,[26, 27] as well as melanoma.[34, 36] Moreover, overexpression of either PHLPP1 or PHLPP2 promotes tumor cell apoptosis and inhibits tumor cell proliferation,[27, 28] whereas knockdown of PHLPP1 or PHLPP2 inhibits tumor cell apoptosis and promotes tumor cell proliferation, supporting the notion of their tumor suppressive function. Indeed, as the brake of AKT, inactivation of PHLPP1 or PHLPP2 promotes activation of AKT and PKC and/or inhibition of Mst (the human homologue of Hippo) to block apoptosis and stimulate cell proliferation.[37, 38] Accordingly, hyperactivation of the PI3K/AKT pathway through co-deletion of PTEN and PHLPP1 also promotes metastasis of prostate cancer.[31] Recent results from Li et al. [39] showed that PHLPP also reduces colorectal cancer cell motility. Clinically, loss of PHLPP1 correlates with lymph node metastasis of gastric cancer. [40] These data strongly implicate the PHLPPs as important regulators of tumor metastasis.

Although both PHLPP1 and PHLPP2 inhibit AKT activation and cell proliferation, our results indicate that their ability to suppress metastasis is not equivalent. First, we found that expression of PHLPP1, but not PHLPP2, was significantly downregulated or lost in metastatic melanoma. Also, the mutations of PHLPP1 in Skin Cutaneous Melanoma (SKCM-TCGA) patients significantly correlate with worse survival, not the mutations of PHLPP2 (Figure S4). Second, overexpression and knockdown of PHLPP1 significantly inhibited and stimulated metastasis, respectively. Although overexpression of PHLPP2 appeared to influence metastasis, there was no significant effect. Third, PHLPP1 and PHLPP2 regulate distinct AKT isoforms; PHLPP1 selectively dephosphorylates AKT2, whereas PHLPP2 dephosphorylates AKT1, consistent with the notion that PHLPP1/AKT2 has a more prominent role in inhibiting metastasis.

PHLPPs encode protein phosphatases belonging to the PP2C family, which contain RA, PH, LRR, PP2C and PDZ binding motif domains.[27, 28, 41] The N-terminal RA domain exists only in PHLPP2 and PHLLP1β, the other domains are common to all PHLPP isoforms. According to a recent study, a mutant form of PHLPP1α lacking the PH domain is unable to dephosphorylate PKC at the hydrophobic motif yet retains the ability to dephosphorylate AKT. In contrast, a truncated form of PHLPP1 in which the PDZ-binding motif is deleted is still able to dephosphorylate AKT but not PKC.[27, 37, 41] Finally, a mutant form of PHLPP1 that lost the ability to dephosphorylate AKT also lost the capacity to inhibit metastasis in melanoma, demonstrating that PHLPP1 phosphatase activity is required for its capacity to regulate metastatic spread.

The firm association of PHLPP1 function with poor metastasis is likely due to its capacity to dephosphorylate the selective substrates Akt2 and Akt3.[27, 28, 41] Indeed, AKT3 was reported to play a role in melanomagenesis,[9] while AKT2 has been reported to promote metastasis in breast,[16–18, 20] ovarian[20] and colorectal cancer, [21] as well as neuroblastoma.[42] Recently,

AKT2 was reported to be a downstream effector of the metabotropic glutamate receptor (Grm1), and to be activated in human melanoma biopsies without PTEN loss. The inhibition of AKT2 also leads to suppression of cell invasion and reduction of tumorigenesis.[43] Although, Cho, et al. proposed that the activation of AKT1 stimulates development of melanoma metastases in mouse $BRAF^{V600E}$/$Cdkn2a^{Null}$ melanoma model,[19] we could not see an effect of AKT1 on melanoma metastasis in our system as well as others in other cancers, [14–18] it is possible that the multiple genetic and model difference results in different phenotype. Indeed, numerous genetic mutations within tumors and hosts not only alter the molecular route to tumorigenesis but also cause different responses to targeted therapy. For example, Martin McMahon and colleagues reported that MK2206 could significantly inhibit the proliferation of WM9 human melanoma[44] and mouse $BRAF^{V600E}$/$PTEN^{Null}$/$CDKN2A^{Null}$ melanoma,[45] but have no effect or modest effect on proliferation of A375, WM793, and 1205Lu melanoma. [44, 45] Similar to Cao et al study that MK2206 inhibited proliferation of all conjunctival melanoma cell lines in a dose-dependent manner,[46] we showed that MK2206 affected activation of AKT2 in A375sm and A375p AKT2 cells and tumor growth and metastasis significantly. However, to our knowledge, a role for AKT2 in the regulation of melanoma metastasis has not been clearly defined. In this study, we demonstrate that AKT2 and AKT3 can not only promote the metastatic potential of melanoma but do so by acting downstream of the metastasis-inhibitory PHLPP1.

We would argue that AKT2 is the more important and relevant regulator of metastasis in melanoma. First, the effect of AKT2 on metastasis is more consequential than that of AKT3. Second, PHLPP2 can dephosphorylate AKT3, yet does not affect metastasis. Third, MK2206 was found to be a potent inhibitor of melanoma metastasis; and although MK2206 was not developed as an isoform-specific AKT inhibitor,[29] our data show that the effects of MK2206 in melanoma cells are focused predominantly on AKT2, with a lesser and no impact on AKT3 and AKT1, respectively. However, we cannot rule out that AKT3 plays a minor role in the regulation of metastasis by PHLPP1.

In summary, we here show that PHLPP1 levels inversely correlate with metastatic phenotype in melanoma, whose metastatic potential can be modulated by manipulating PHLPP1 expression. Moreover, the metastasis-suppressive function of PHLPP1 is mediated predominantly through inhibition of AKT2. Our data raise the possibility that PHLPP1 status could serve as a prognostic marker of melanoma progression and suggest that the PHLPP1/AKT2 axis provides new targets for treatment of metastatic melanoma.

Materials and Methods

Reagents, cell lines, and culture.

Plasmids: pcDNA3-HA-PHLPP1, pcDNA3-HA-PHLPP2, pcDNA3-HA-PHLPP1-ΔC, pcDNA3-HA-PHLPP1-ΔPH, pBabe myrHA Akt2, pBabe myrHA Akt3, pBabe myr-Flag-Akt1 or pLNCX myrHA AKT1 were provided by Addgene (Cambridge, MA). PHLPP1, PHLPP2, AKT1, AKT2 and AKT3 shRNAs expressing plasmids were purchased from Open Biosystems (GE Dharmacon, Lafayette, CO). CRISPR/cas9 knockout vectors for AKT1, AKT2 or AKT3 were constructed using Leonti CRISPR V2 vector (addgene, Cambridge MA). Antibodies: anti-PHLPP1, anti-PHLPP2 antibodies were purchased from Novus

(Littleton, CO), anti-phospho-AKT (Ser473), anti-phospho-AKT (Thr308), anti-AKT, anti-phospho-ERK 1/2 (Thr202/Tyr204), anti-ERK1/2, anti-akt1, anti-akt2 and anti-akt3 antibodies and ELISA kits were purchased from Cell Signaling (Danvers, MA, USA); anti-β-actin antibody was purchased from Santa Cruz (Dallas, TX). The B16F1, 1205Lu, sk-mel-28 and Malme 3M cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). WM4007, WM88, WM3899, WM9, WM3928, WM46, WM3912, WTH202, WM3918, WM983B, WLH6215, WRL159, WM4077 and WM858 were gifted by Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA). A375p and A375sm were a gift from Dr. Isaiah Fidler (M.D. Anderson Medical Center, Houston, TX); both derived from the same human melanoma tumor with different metastatic potential. A375p is the parent cell line with poorly metastatic potential, A375sm were developed from A375P with highly (super) metastatic potential. The stable expressing cells were established through transfection using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and selected by antibiotics G418 or puromycin (Sigma, St. Louis, MO).

Protein immunoblot analyses.

Immunoblots were performed on lysates generated from cultured cells and tissues solubilized in RIPA buffer.[47–50]

Cell motility and invasion assays.

The ability of cell motility and invasion were measured as described before.[47] The experiment was performed in quadruplicate and repeated three times.

Cell proliferation assay.

^{3}H-thymidine incorporation assay and CK8 kit were used for measurement of cell growth. The experiment was performed in quadruplicate and repeated three times.[47–49]

Enzyme-linked immunosorbent assay (ELISA).

Protein level of phospho-AKT 1 (Ser473), phospho-AKT 2(Ser474), phospho-AKT 3(Ser472), phospho-AKT (Thr308), total-AKT1, total-AKT2, total-AKT3, phospho-ERK 1/2 (Thr202/Tyr204) and total-ERK1/2 were measured by ELISA assays using PathScan® Phospho-AKT1 (Ser473), PathScan® Phospho -Akt2 (Ser474), PathScan® Phospho –Akt3 (Ser472), PathScan® Phospho -Akt (Thr308), PathScan® Total Akt1, PathScan® Total Akt2, PathScan® Total Akt3, PathScan® Phospho –Erk1/2 (Thr202/Tyr204) and PathScan® Total Erk1/2 Sandwich ELISA Kits purchased from Cell Signaling (Danvers, MA, USA). The ELISA assays were performed as a described protocol by the manufacturer. The experiment was performed in quadruplicate and repeated three times.

Soft agar colony formation assay.

We plated aliquots of logarithmically growing cells (1×10e3 cells) as a single-cell suspension in 0.35% agarose in MEM containing 10% FBS and laid on a 0.6% agarose previously gelled layer with the same complete media in six-well dishes. After a 20-day incubation, 1 ml of 0.5 mg/ml p-iodonitrotetrazolium violet (Sigma) was added to each well

and incubated overnight. Colonies with a diameter more than 100 μm were counted. The experiment was performed in triplicate and repeated three times.[50]

Experimental and spontaneous metastasis assays.

Cells were intravenously injected via tail vein or footpad into 4 - 6-week-old male mice (n = 6 to 10). There were three different parent cell lines: B16F1 cells were injected at 5×10^5 into C57/BL6-cBrd or 1×10^5 into athymic nude; human melanoma A375p and A375sm cells were injected via tail vein or footpad into SCID or SNG mice at 1×10^6. Tumor numbers were obtained by visual inspection of tissues in mice euthanized 3 to 10 weeks after transplantation, and micrometastases were counted by pathologist's evaluation after dissection of the lung .[47–49]

Immunohistochemistry:

Lung tissues were fixed in 10% buffered formalin solution (pH7.2) for 16 h, and serially sectioned to 15 μm at 20°C. Immunohistochemistry was performed as described.[47,48] Immunoreactivity scores were analyzed using ImageScope V 10.0 software from Aperio Technologies (Vista, CA). The size of metastases was quantified by ImageJ software and analyzed using Statgraphics software.

Preclinical drugs treatments.

A375sm or A375p cells stably expressing Myr-Akt2 cells (200 ul, 1×10e6) were transplanted into 6 to 10 male SNG mice between 4 and 6 weeks of age either intravenously (IV) or subcutaneously (SQ). Mice harboring the inoculated tumor cells were treated with 60 mg/kg or 120 mg/kg of the AKT inhibitor MK-2206 three times a week by oral gavage either immediately or 7 days after transplantation of tumor. Animals had to be held for between 4 or 5 weeks post-transplantation to achieve valid metastasis analyses. All mouse procedures were conducted following the NIH guidelines [the animal proposal LCBG023, approval by NCI-Bethesda Animal Care and Use Committee (ACUC)].

Analyses of clinical outcome data.

Clinical follow-up and gene expression data sets were obtained from publicly available data set (GSE7553) in Gene Expression Omnibus (GEO).

Statistics.

Statistical analyses were performed using unpaired t-test (two-tailed with mean ± s.e.m.) or Student's t-test (two-tailed with mean ± s.d.) for all column data sets using GraphPad Prism 6 software. The p values of less than 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the funding from the NIH intramural research program. We thank Drs. Miriam Anver, Julia Zhang and Hong Sheng for assistance with tissue microarray and immunohistochemistry.

References

1. Hunter T Tyrosine phosphorylation: thirty years and counting. Curr Opin Cell Biol 2009; 21(2):140–146. [PubMed: 19269802]
2. Alonso A, Sasin J, Bottini N, Friedberg I, Friedberg I, Osterman A et al. Protein tyrosine phosphatases in the human genome. Cell 2004; 117(6):699–711. [PubMed: 15186772]
3. Julien SG, Dubé N, Hardy S, Tremblay ML. Inside the human cancer tyrosine phosphatome. Nat Rev Cancer 2011; 11(1):35–49. [PubMed: 21179176]
4. Blume-Jensen P, Hunter T. Oncogenic kinase signaling. Nature 2001; 411(6835):355–365. [PubMed: 11357143]
5. Ostman A, Hellberg C, Böhmer FD. Protein-tyrosine phosphatases and cancer. Nat Rev Cancer 2006; 6(4):307–320. [PubMed: 16557282]
6. Brazil DP, Hemmings BA. Ten years of protein kinase B signaling: a hard Akt to follow. Trends Biochem Sci 2001; 26(11):657–664. [PubMed: 11701324]
7. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. Genes Dev 1999; 13(22):2905–2927. [PubMed: 10579998]
8. Davies MA, Stemke-Hale K, Lin E, Tellez C, Deng W, Gopal YN et al. Integrated Molecular and Clinical Analysis of AKT Activation in Metastatic Melanoma. Clin Cancer Res 2009; 15(24):7538–7546. [PubMed: 19996208]
9. Stahl JM, Sharma A, Cheung M, Zimmerman M, Cheng JQ, Bosenberg MW et al. Deregulated Akt3 activity promotes development of malignant melanoma. Cancer Res 2004; 64(19):7002–7010. [PubMed: 15466193]
10. Dai DL, Martinka M, Li G. Prognostic significance of activated Akt expression in melanoma: a clinicopathologic study of 292 cases. J Clin Oncol 2005; 23(7):1473–1482. [PubMed: 15735123]
11. Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK et al. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell 1995; 81(5):727–736. [PubMed: 7774014]
12. Scheid MP, Woodgett JR. PKB/AKT: functional insights from genetic models. Nat Rev Mol Cell Biol 2001; 2(10):760–768. [PubMed: 11584303]
13. Woodgett JR. Recent advances in the protein kinase B signaling pathway. Curr Opin Cell Biol 2005; 17(2):150–157. [PubMed: 15780591]
14. Bellacosa A, Testa JR, Moore R, Larue L. A portrait of AKT kinases: human cancer and animal models depict a family with strong individualities. Cancer Biol Ther 2004; 3(3):268–275. [PubMed: 15034304]
15. Stambolic V, Woodgett JR. Functional distinctions of protein kinase B/Akt isoforms defined by their influence on cell migration. Trends Cell Biol 2006, 16(9):461–466. [PubMed: 16870447]
16. Dillon RL, Marcotte R, Hennessy BT, Woodgett JR, Mills GB, Muller WJ. Akt1 and Akt2 play distinct roles in the initiation and metastatic phases of mammary tumor progression. Cancer Res 2009; 69:5057–5064. [PubMed: 19491266]
17. Hutchinson J, Jin J, Cardiff RD, Woodgett JR, Muller WJ. Activation of Akt (protein kinase B) in mammary epithelium provides a critical cell survival signal required for tumor progression. Mol Cell Biol 2001; 21:2203–2212. [PubMed: 11238953]
18. Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Toker A. Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. Mol Cell 2005; 20:539–550. [PubMed: 16307918]
19. Cho JH, Robinson JP, Arave RA, Burnett WJ, Kircher DA, Chen G et al. AKT1 Activation Promotes Development of Melanoma Metastases. Cell Rep. 2015; 13(5):898–905. [PubMed: 26565903]
20. Arboleda MJ, Lyons JF, Kabbinavar FF, Bray MR, Snow BE, Ayala R et al. Overexpression of AKT2/Protein Kinase Bβ leads to up-regulation of β1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. Cancer Res 2003; 63:196–206. [PubMed: 12517798]

21. Rychahou PG, Kang J, Gulhati P, Doan HQ, Chen LA, Xiao SY et al. Akt2 overexpression plays a critical role in the establishment of colorectal cancer metastasis. Proc Natl Acad Sci USA 2008; 105(51):20315–20320. [PubMed: 19075230]
22. Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA et al. The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. Proc Natl Acad Sci USA 1998; 95(23):13513–13518. [PubMed: 9811831]
23. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 1997; 275(5308): 1943–1947. [PubMed: 9072974]
24. Song MS, Salmena L, Pandolfi PP. The functions and regulation of the PTEN tumour suppressor. Nat Rev Mol Cell Biol 2012; 13(5):283–296. [PubMed: 22473468]
25. Tamura M, Gu J, Matsumoto K, Aota S, Parsons R, Yamada KM. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science 1998; 5;280(5369):1614–1617. [PubMed: 9616126]
26. Molina JR, Agarwal NK, Morales FC, Hayashi Y, Aldape KD, Cote G et al. PTEN, NHERF1 and PHLPP form a tumor suppressor network that is disabled in glioblastoma. Oncogene 2012; 31(10): 1264–1274. [PubMed: 21804599]
27. Gao T, Furnari F, and Newton AC. PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. Mol Cell 2005; 18(1):13–24. [PubMed: 15808505]
28. Brognard J, Sierecki E, Gao T, Newton AC. PHLPP and a second isoform, PHLPP2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms. Mol Cell 2007; 25(6): 917–931. [PubMed: 17386267]
29. Hirai H, Sootome H, Nakatsuru Y, Miyama K, Taguchi S, Tsujioka K et al. MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. Mol Cancer Ther 2010; 9(7):1956–1967. [PubMed: 20571069]
30. Liu J, Weiss HL, Rychahou P, Jackson LN, Evers BM, Gao T. Loss of PHLPP expression in colon cancer: role in proliferation and tumorigenesis. Oncogene 2009; 28(7):994–1004. [PubMed: 19079341]
31. Chen M, Pratt CP, Zeeman ME, Schultz N, Taylor BS, O'Neill A et al. Identification of PHLPP1 as a tumor suppressor reveals the role of feedback activation in PTEN-mutant prostate cancer progression. Cancer Cell 2011; 20(2):173–186. [PubMed: 21840483]
32. Nitsche C, Edderkaoui M, Moore RM, Eibl G, Kasahara N, Treger J et al. The phosphatase PHLPP1 regulates Akt2, promotes pancreatic cancer cell death, and inhibits tumor formation. Gastroenterology 2012; 142(2):377–387. [PubMed: 22044669]
33. O'Hayre M, Niederst M, Fecteau JF, Nguyen VM, Kipps TJ, Messmer D et al. Mechanisms and consequences of the loss of PHLPP1 phosphatase in chronic lymphocytic leukemia (CLL). Leukemia 2012; 26(7):1689–1692. [PubMed: 22237780]
34. Dong L, Jin L, Tseng HY, Wang CY, Wilmott JS, Yosufi B et al. Oncogenic suppression of PHLPP1 in human melanoma. Oncogene 2014;33(39):4756–4766. [PubMed: 24121273]
35. O'Neill AK, Niederst MJ, Newton AC. Suppression of survival signaling pathways by the phosphatase PHLPP. FEBS J. 2013; 280(2):572–583 [PubMed: 22340730]
36. Mann MB, Black MA, Jones DJ, Ward JM, Yew CC, Newberg JY et al. Transposon mutagenesis identifies genetic drivers of Braf (V600E) melanoma. Nat Genet 2015, 47(5):486–495. [PubMed: 25848750]
37. Gao T, Brognard J, Newton AC. The phosphatase PHLPP controls the cellular levels of protein kinase C. J Biol Chem 2008; 283(10):6300–6311. [PubMed: 18162466]
38. Qiao M, Wang Y, Xu X, Lu J, Dong Y, Tao W et al. Mst1 is an interacting protein that mediates PHLPPs' induced apoptosis. Mol Cell 2010; 38(4):512–523. [PubMed: 20513427]
39. Li X, Stevens PD, Liu J, Yang H, Wang W, Wang C et al. PHLPP is a negative regulator of RAF1, which reduces colorectal cancer cell motility and prevents tumor progression in mice. Gastroenterology 2014; 146(5):1301–1312. [PubMed: 24530606]

40. Wang Z, Shu H, Wang Z, Li G, Cui J, Wu H et al. Loss expression of PHLPP1 correlates with lymph node metastasis and exhibits a poor prognosis in patients with gastric cancer. J Surg Oncol 2013; 108(7):427–432. [PubMed: 24037758]

41. Brognard J, Newton AC. PHLiPPing the switch on Akt and protein kinase C signaling. Trends Endocrinol Metab 2008; 19(6):223–230. [PubMed: 18511290]

42. Qiao J, Lee S, Paul P, Qiao L, Taylor CJ, Schlegel C et al. Akt2 regulates metastatic potential in neuroblastoma. PLoS One 2013;8(2):e56382. [PubMed: 23468863]

43. Shin SS, Wall BA, Goydos JS, Chen S. AKT2 is a downstream target of metabotropic glutamate receptor 1 (Grm1). Pigment Cell Melanoma Res 2010; 23(1):103–111. [PubMed: 19843246]

44. Silva JM, Bulman C, McMahon M. BRAFV600E cooperates with PI3K signaling, independent of AKT, to regulate melanoma cell proliferation. Mol Cancer Res. 2014;12(3):447–463. [PubMed: 24425783]

45. Marsh Durban V, Deuker MM, Bosenberg MW, Phillips W, McMahon M. Differential AKT dependency displayed by mouse models of BRAFV600E-initiated melanoma. J Clin Invest. 2013;123(12):5104–5118. [PubMed: 24200692]

46. Cao J, Heijkants RC, Jochemsen AG, Dogrusöz M, de Lange MJ, van der Velden PA et al. Targeting of the MAPK and AKT pathways in conjunctival melanoma shows potential synergy. Oncotarget. 2016;8(35):58021–58036. [PubMed: 28938534]

47. Yu Y, Merlino G. Constitutive c-Met signaling through a nonautocrine mechanism promotes metastasis in a transgenic transplantation model. Cancer Res 2002; 62(10):2951–2956. [PubMed: 12019177]

48. Yu Y, Zeng P, Xiong J, Liu Z, Berger SL, Merlino G. Epigenetic drugs can stimulate metastasis through enhanced expression of the pro-metastatic Ezrin gene. PLoS One 2010; 5(9):e12710. [PubMed: 20856924]

49. Yu Y, Khan J, Khanna C, Helman L, Meltzer PS, Merlino G. Expression profiling identifies the cytoskeletal organizer ezrin and the developmental homeoprotein Six-1 as key metastatic regulators. Nat Med 2004; 10(2):175–181. [PubMed: 14704789]

50. Yan J, Yu Y, Wang N, Chang Y, Ying H, Liu W et al. LFIRE-1/HFREP-1, a liver-specific gene, is frequently downregulated and has growth suppressor activity in hepatocellular carcinoma. Oncogene 2004; 23, 1939–1949. [PubMed: 14981537]

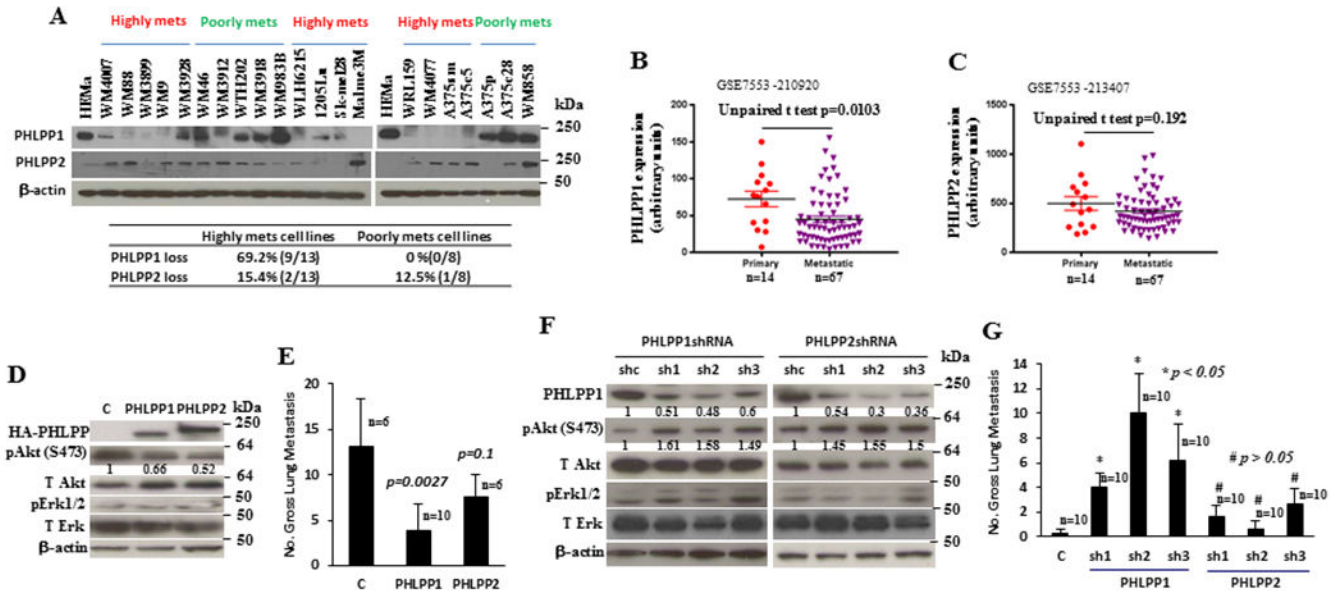

Figure 1. PHLPP1 suppresses metastasis in human melanoma.

(**A**) The protein level of PHLPP1 and PHLPP2 was analyzed by western blot in highly and poorly metastatic human melanoma cell lines. (B, C) Expression of PHLPP1(B) and PHLPP2 (C) in primary and metastatic melanoma tumors in the GSE7553 dataset. (D) The western blot analysis of highly metastatic melanoma A375sm cells transfected with PHLPP1, PHLPP2 or empty vector control (c). (E) Gross pulmonary metastases of A375sm cells transfected with PHLPP1 or PHLPP2 expression vectors were determined using orthotopic footpad transplantation in NSG mice. (F) The western blot analysis of poorly metastatic melanoma A375p cells transfected with shRNAs for PHLPP1 or PHLPP2. (G) Gross pulmonary metastasis of A375p cells transfected with shRNAs for PHLPP1 or PHLPP2 was determined using orthotopic footpad transplantation in NSG mice. Knockdown of endogenous PHLPP1 by shRNAs enhanced metastatic potential in A375p cells ($p<0.05$). shc, vector control; sh1, sh1, and sh3 are different shRNA constructs. #, no statistical difference ($p>0.05$); *, statistical significant difference ($p<0.05$).

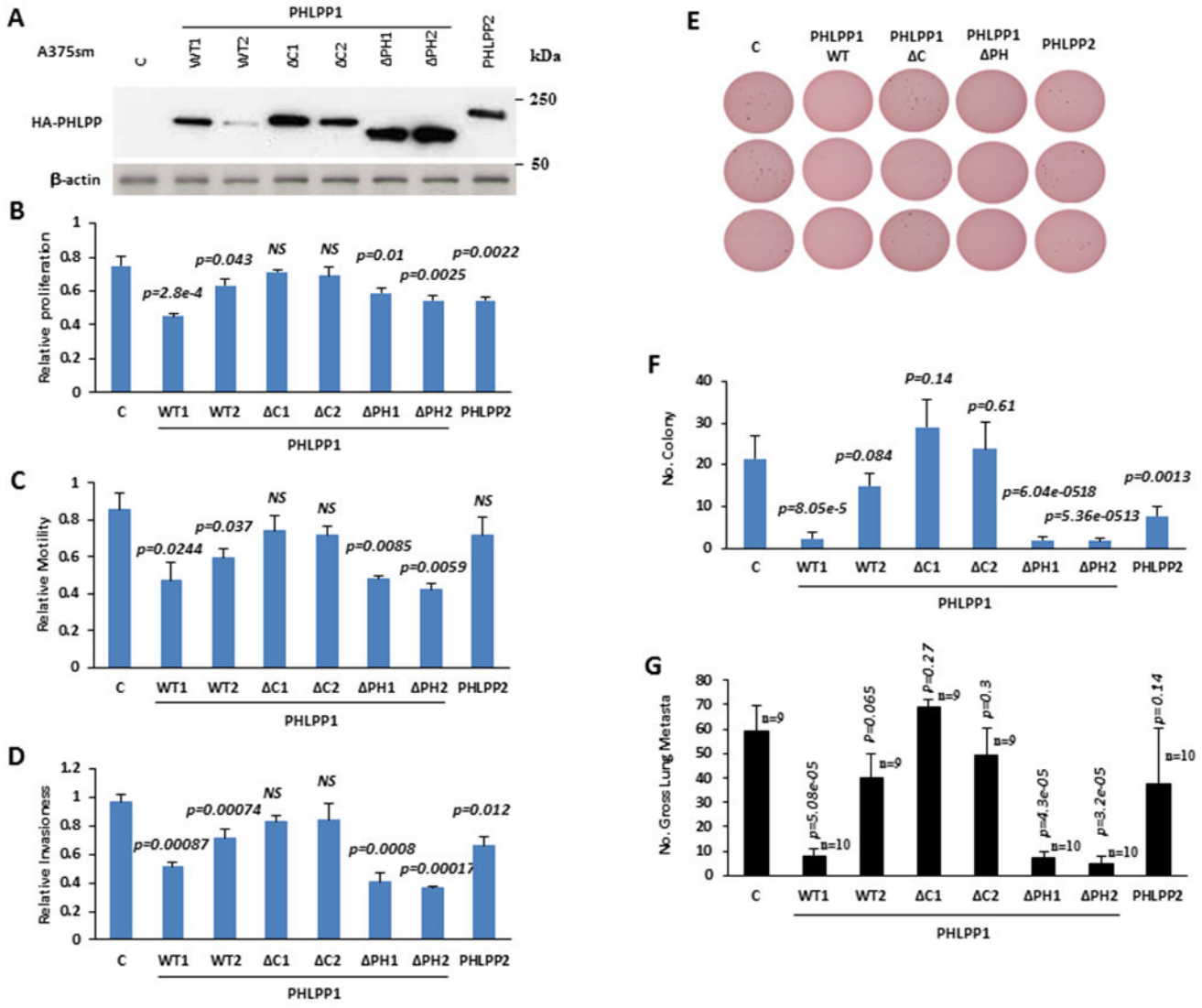

Figure 2. PHLPP1 inhibits metastasis through its phosphatase activity.

(A) Western blot analysis of A375sm cells transfected with vector (c), PHLPP1 (WT1, WT2), PHLPP1ΔC (ΔC1, ΔC2), PHLPP1ΔPH (ΔPH1, ΔPH2) or PHLPP2 expressing constructs. (B-G) A375sm cells transfected with the various PHLPP1- and PHLPP2-expressing constructs described in (A) were analyzed with CCK8 to assess cell proliferation (B), with transwell to determine the cell motility (C), with matrigel coated transwell to determine the cell invasiveness, with soft-agar to determine anchorage-independent colony formation (E, F) and with an experimental metastatic assay by tail vein injection in SCID mice to determine the gross pulmonary metastasis (G). NS, not significant.

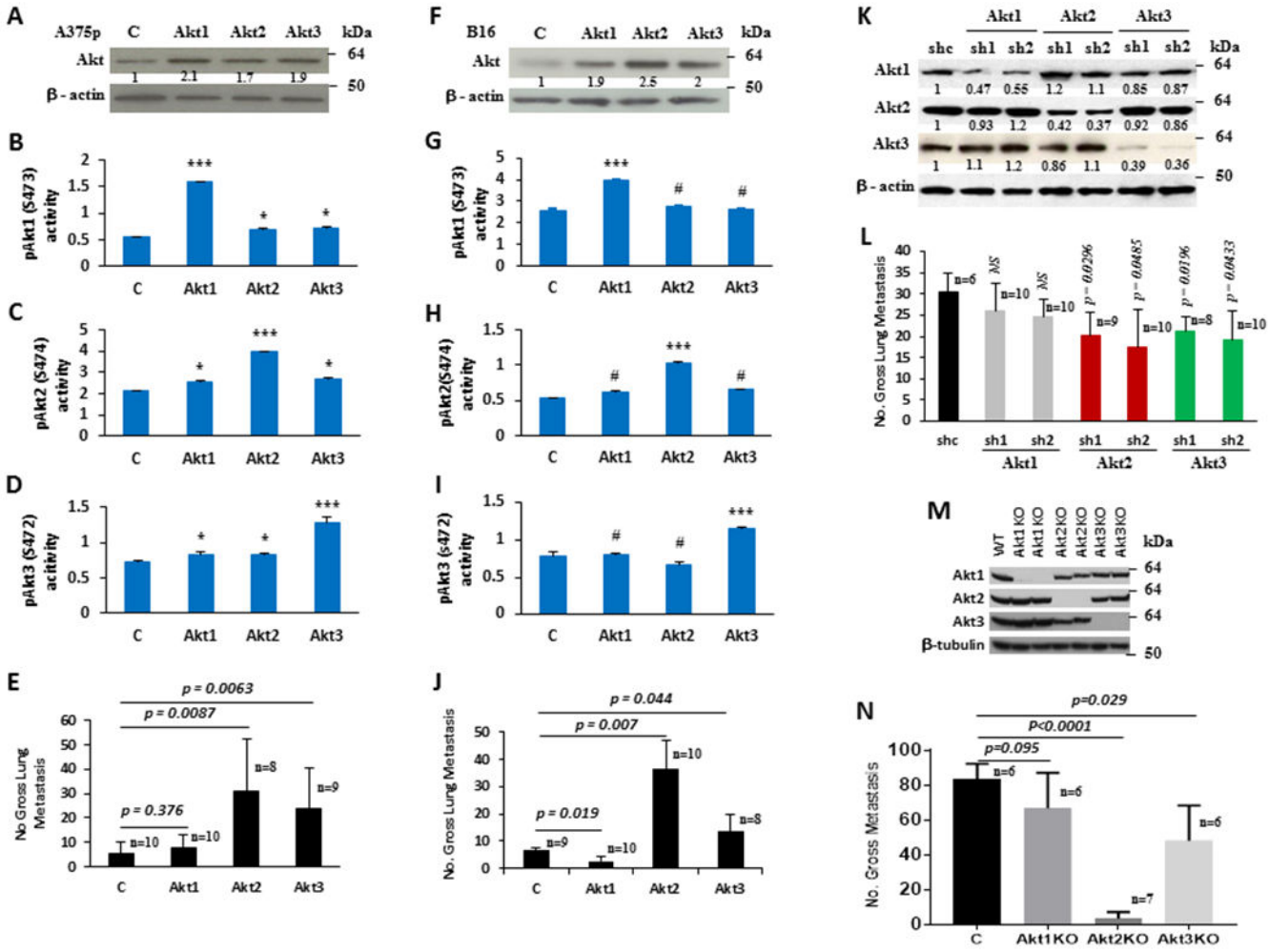

Figure 3. Constitutive expression of activated AKT2, and to a lesser extent AKT3, promotes metastasis in melanoma.

(A-E) Overexpression of activated AKT2 and AKT3 promotes metastasis in human A375p cells. Pan-AKT expression was analyzed by Western blot of A375p cells transfected with myr-AKT1, myr-AKT2 or myr-AKT3 (A). A375p cell transfected with the constructs described in (A) were analyzed by ELISA to determine phosphorylation of AKT1 (B), AKT2 (C) and AKT3 (D), and by experimental metastasis assay using tail vein injection to determine the gross pulmonary metastasis (E). c, empty vector control. Compared to vector control, total of Akt1, Akt2 and Akt3 were increased by 2.1, 1.7 and 1.9 fold respectively; phosphorylated Akt1, Akt2 and Akt3 were significantly enhanced by 2.9, 1.9 and 1.8 fold respectively. (F-J) Overexpression of activated AKT2 and AKT3 promotes metastasis in mouse B16F1 cells. Pan-AKT expression was analyzed by western blot of B16 cells transfected with myr-AKT1, myr-AKT2 or myr-AKT3 (F). B16 cells transfected with the constructs described in (F) were analyzed by ELISA to determine the phosphorylation of AKT1 (G), AKT2 (H) and AKT3 (I), and by experimental metastasis assay using tail vein injection to determine the gross pulmonary metastasis (J). c, empty vector control. Compared to vector control, total of Akt1, Akt2 and Akt3 were increased by 1.9, 2.5 and 2 fold respectively; phosphorylated Akt1, Akt2 and Akt3 were significantly enhanced by 1.6,

2 and 1.7 fold respectively. (K, L) Knockdown of AKT2 and AKT3 inhibits metastasis in human A375sm cells. A375sm cell transfected with shRNA for AKT1, AKT2 and AKT3 were analyzed by western blot with Akt isoform-specific anti-Akt1, Akt2 and Akt3 antibodies (K); gross pulmonary metastasis was determined by experimental metastasis assay using tail vein injection in NSG mice (L). shc, vector control; sh1 and sh2, different shRNAs of AKT1, AKT2 or AKT3. β-actin as an internal control. (M) A375sm cell cotransfected with CRISPR/cas9 and HDR plasmid for specifically knockout of AKT1, AKT2, and AKT3 were analyzed by western blot with Akt isoform-specific anti-AKT1, AKT2 and AKT3 antibodies. β-tubulin as an internal control. (N) Gross pulmonary metastasis was determined by experimental metastasis assay using tail vein injection of knockout of AKT1 (Akt1KO), AKT2 (Akt2KO) and AKT3 (Akt3KO) cells in NSG mice.

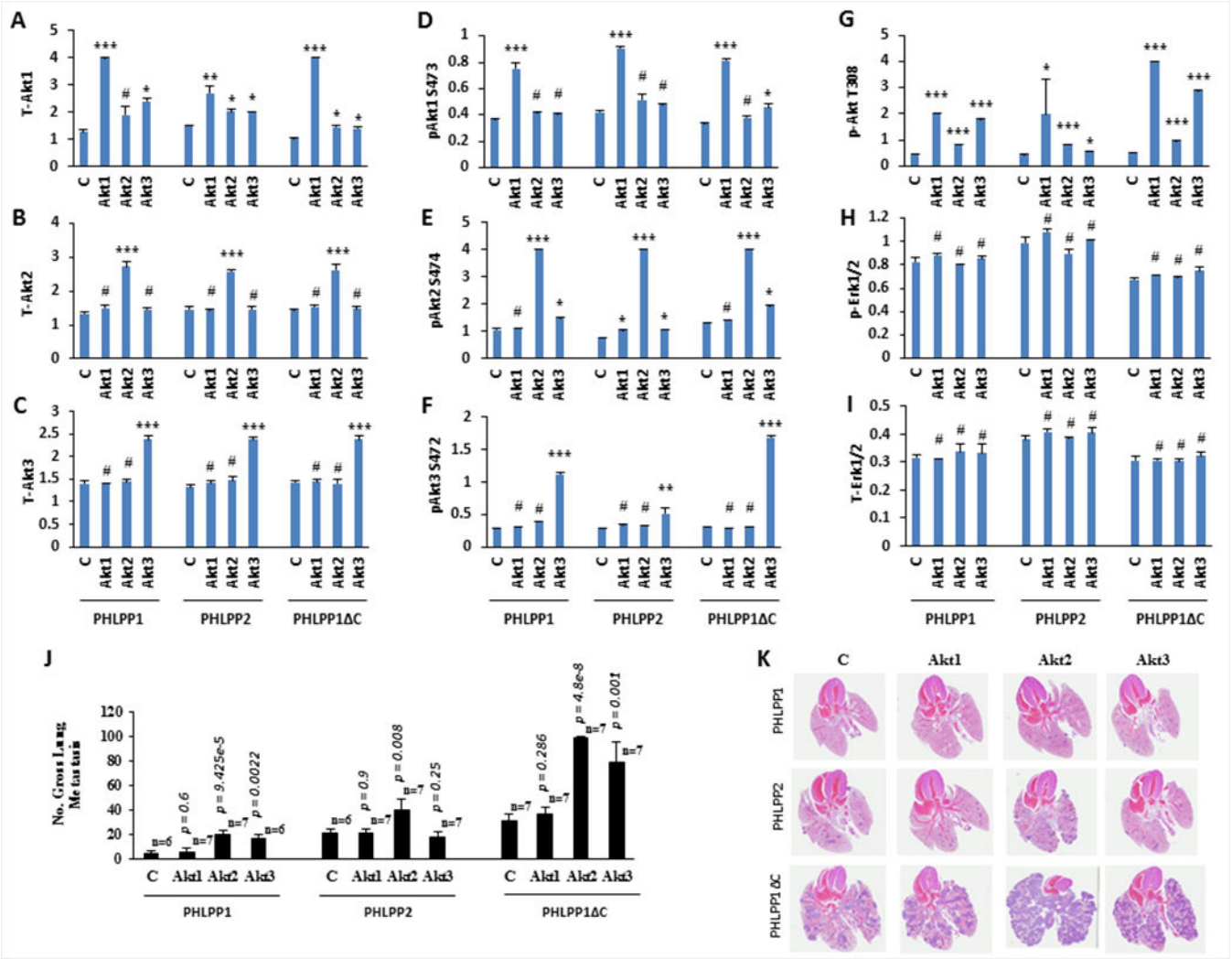

Figure 4. Activated AKT2 and AKT3 can rescue PHLPP1-mediated inhibition of metastasis in melanoma.

(A-I) ELISA analysis of stable A375sm expressing PHLPP1, PHLPP1ΔC and PHLPP2 cells transfected with myr-Akt1, myr-AKT2 or myr-AKT3 plasmids was performed to determine protein levels of total Akt1 (A), total Akt2 (B) and total Akt3 (C), phosphorylated Akt1 (D), phosphorylated Akt2 (E), phosphorylated Akt3 (F), phosphorylated Akt-Thr-308 (G), phosphorylated Erk1/2 (H), total Erk1/2 (I); c, empty vector control; #, no statistical difference; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (J) Gross pulmonary metastases of A375sm cells stably expressing PHLPP1, PHLPP1ΔC or PHLPP2 cells transfected with myr-AKT1, myr-AKT2 or myr-AKT3 constructs were determined using tail vein injection in SCID mice. c, empty vector control. (K) Representative histopathology with H&E staining of lung sections with metastases from mice inoculated with stable A375sm expressing PHLPP1, PHLPP1ΔC or PHLPP2 cells transfected with myr-AKT1, myr-AKT2 or myr-AKT3 constructs. c, empty vector control.

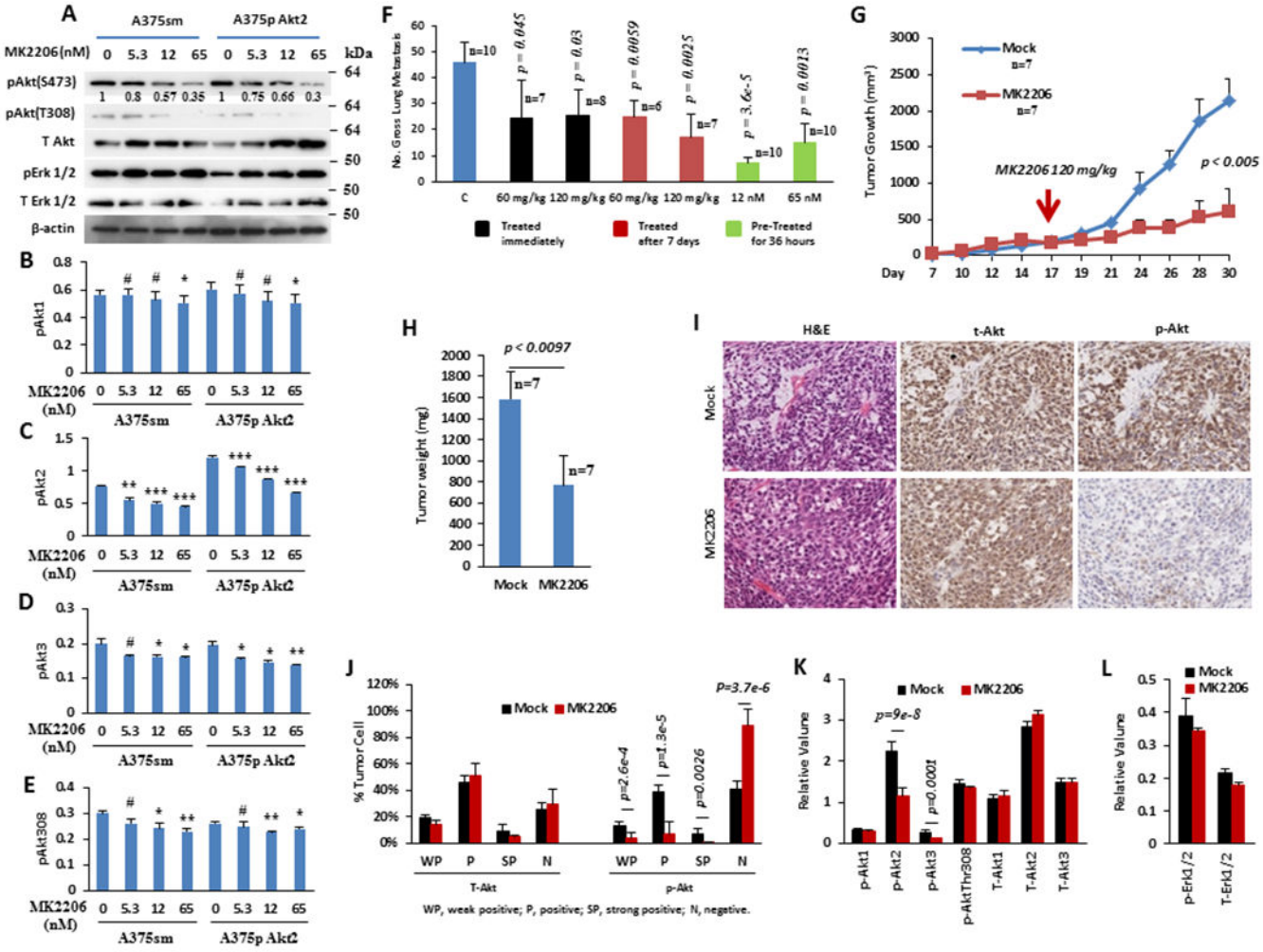

Figure 5. Inhibition of AKT2 by MK2206 blocks in vivo tumor growth and metastasis.

(A) Whole cell lysates from A375sm cells and stable A375p-myr-AKT2 (A375p Akt2) cells treated with various doses of MK2206 were analyzed by western blot. (B-E) ELISA analysis of A375sm and stable A375p myr-AKT2 cells treated with different doses of MK2206 was performed to determine protein levels of phosphorylated AKT1 (B), phosphorylated AKT2 (C), phosphorylated AKT3 (D), and phosphorylated AKT-Thr-308 (E). #, no statistical difference; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (F) Gross pulmonary metastases from mice treated with MK2206 or from pretreated cultured A375p Akt2 cells with either 12 nM or 65 nM MK2206 for 36 hours in cell culture (green color). Mice harboring A375p cells stably transfected with the myr-AKT2 expression vector (A375p Akt2) were treated with two different doses of MK2206 immediately (black color) or 7 days after cells injection (red color). After pretreatment with MK2206 for 36 hours, A375p Akt2 cells were injected into NSG mice by tail vein. c, mock control. (G-H) Tumor growth curve (G) and tumor weight (H) of A375sm cells in xenograft NSG mice with/without treatment of MK2206. (I) Representative histopathology (H&E staining) or immunohistochemical staining in metastases derived from mice bearing A375sm cells treated with MK2206. H&E, hematoxylin and eosin staining; t-Akt, immunohistochemical staining with the anti-AKT

antibody; p-Akt, immunohistochemical staining with anti-p-AKT antibody 20X. (J) Immunoreactivity score of total AKT (T-Akt) and phosphorylated AKT (p-Akt) expression in metastases from mice treated with MK2206. Quantitative scores analyzed using ImageScope V10.0 software from Aperio Technologies, presenting the percentage of cells in strongly positive, positive, weakly positive and negative for immunohistochemical staining with an anti-AKT or phosphorylated AKT antibody. (K-L) Whole tissue lysates from tumors in mice treated with MK2206 were analyzed by ELISA to determine protein levels of phosphorylated AKT1 (p-Akt1), phosphorylated AKT2 (p-Akt2), phosphorylated AKT3 (p-Akt3), phosphorylated AKT-Thr-308 (p-AktThr308), and phosphorylated Erk1/2 (p-Erk1/2), total Erk1/2 (T-Erk1/2), total AKT (T-Akt1), total AKT2 (T-Akt2) and total AKT3 (T-Akt3).