PDK1 and SGK3 Contribute to the Growth of BRAF-Mutant Melanomas and Are Potential Therapeutic Targets

Marzia Scortegagna¹, Eric Lau¹, Tongwu Zhang², Yongmei Feng¹, Chris Sereduk³, Hongwei Yin³, Surya K. De¹, Katrina Meeth⁴,⁵, James T. Platt⁴,⁵, Casey G. Langdon⁴,⁵, Ruth Halaban⁴, Maurizio Pellecchia¹, Michael A. Davies⁶, Kevin Brown², David F. Stern⁵, Marcus Bosenberg⁴,⁵, and Ze’ev A. Ronai¹

Abstract

Melanoma development involves members of the AGC kinase family, including AKT, PKC, and, most recently, PDK1, as elucidated recently in studies of Braf::Pten mutant melanomas. Here, we report that PDK1 contributes functionally to skin pigmentation and to the development of melanomas harboring a wild-type PTEN genotype, which occurs in about 70% of human melanomas. The PDK1 substrate SGK3 was determined to be an important mediator of PDK1 activities in melanoma cells. Genetic or pharmacologic inhibition of PDK1 and SGK3 attenuated melanoma growth by inducing G1 phase cell-cycle arrest. In a synthetic lethal screen, pan-PI3K inhibition synergized with PDK1 inhibition to suppress melanoma growth, suggesting that focused blockade of PDK1/PI3K signaling might offer a new therapeutic modality for wild-type PTEN tumors. We also noted that responsiveness to PDK1 inhibition associated with decreased expression of pigmentation genes and increased expression of cytokines and inflammatory genes, suggesting a method to stratify patients with melanoma for PDK1-based therapies. Overall, our work highlights the potential significance of PDK1 as a therapeutic target to improve melanoma treatment.

Introduction

Approximately 70% of melanomas with mutated BRAF also exhibit the inactivation of the tumor suppressor PTEN, resulting in constitutive activation of the PI3K signaling pathway (1). Phosphoinositide-dependent kinase 1 (PDK1), an immediate downstream effector of PI3K, is a master kinase able to phosphorylate more than 20 members of the AGC kinase family, which includes PKA, AKT, PKC, p70S6k, and SGK (2, 3). The relationship between PDK1 and PTEN was first revealed by the demonstration that the lethality of Pten deficiency in flies was rescued by deletion of Pdk1, establishing PDK1 as the main downstream effector of PI3K (4). Recently, we investigated the role of PDK1 in melanomas using a mouse model in which expression of mutated BRAF (BrafV600E) and deletion of Pten is conditionally and specifically activated in melanocytes (5).

Using this model, we showed that genetic inactivation or pharmacologic inhibition of PDK1 delays melanoma development and metastasis. However, wild-type (WT) PTEN is expressed in a sizable fraction (~70%) of BRAF-mutant human melanomas (1, 6, 7), and the role of PDK1 in the progression of such melanomas is unknown. Here, we used genetic and pharmacologic models to show that PDK1 plays an even more significant role in the development of WT Pten, BrafV600E mouse and human melanomas, compared with the Pten-deficient, BrafV600E melanomas.

Although studies in several cancer types suggest that AKT is the main downstream effector of the PI3K/PDK1 signaling pathway, increasing evidence indicates that additional factors are equally important (8–10). For example, the overexpression of AKT in PDK1-knockout (KO) cancer cells was demonstrated to be insufficient to restore the malignant phenotype (11). The 3 isoforms of the SGK family of AGC kinases, SGK1, SGK2, and SGK3, are also activated by the PI3K/PDK1 signaling pathway. SGKs exhibit similar substrate specificity to AKT, and both kinases influence the activity of proteins involved in cell growth, survival, and migration (12, 13). Several studies have demonstrated important roles for the SGK isoforms in PI3K signaling in both physiologic and pathologic conditions. SGK1 and SGK3 are ubiquitously expressed, whereas SGK2 is restricted to the kidney, pancreas, liver, and brain. Given the function of SGK1 and SGK3 in cell proliferation and survival, it is not surprising that they have been shown to be involved in the growth of several cancers (14, 15). However, their contribution in melanoma remains unclear.

In this study, we identify SGKs as key mediators of PDK1 activity in melanoma and demonstrate the importance of the PDK1/SGK signaling axis in the growth of PTEN WT melanomas.
We also demonstrate that PI3K inhibitor can synergize with PDK1 inhibitors in suppressing melanoma growth and point to possible means for the stratification of human BRAFV600E PTEN WT tumors for PDK-targeted therapies.

Materials and Methods

Primary melanoma cells and human melanoma cell lines
Murine melanoma cells Sanford Burnham Melanoma A2 (SBM-A2) and SBM-A3 were derived from primary cutaneous lesions from BratV600E::Cdkn2a/-/- mice. Tumors were cut in small parts and digested with collagenase (10 mg/mL; Sigma) for 1 hour at 37°C and filtered through a 100-µm nylon cell strainer (BD Falcon). Cells were resuspended in DMEM supplemented with 10% FBS and penicillin/streptomycin and incubated at 37°C. Once established, cell lines were passaged twice before use in experiments. The mouse YUMM1.5 and YUMM1.9 and the human 501Mel and UACC903 cell lines were maintained in DMEM medium supplemented with 10% FBS and penicillin/streptomycin. Information on the genotype and origin of the cell lines used is shown in Table S2.

Activation of the 6-Hydroxymatairesinol (4-HT) was prepared at 50 µg/mL in DMSO and 10 µL was applied to the dorsal skin on postnatal days 1, 3, and 5 using a small paintbrush.

Histologic analyses
Tumors sections were fixed overnight in Z-Fix (buffered zinc formalin fixative, Anatech) at 4°C. Sections were then washed twice with PBS and processed for paraffin embedding. Paraffin blocks were sliced at 5 µm and sections were stained with hematoxylin and cosin (H&E).

Antibodies and reagents
The following antibodies were purchased from Cell Signaling Technologies: pNDRG1, pPDK1, pAKT308, pAKT473, pGSK3b, pP70S6K (Thr 389), pS6K (Ser 235/236), PRAS40, SGK1, PKC, and tubulin were purchased from Santa Cruz Biotechnology. 4-HT and antibodies against S100 were purchased from Sigma. Synthesis of the primer sequences were:

<table>
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<td>H3.3A</td>
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<td>Bladder</td>
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<tr>
<td>Lung</td>
<td>5'-gtccacgctgctgctcatac-3'</td>
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Gene silencing and transfection
shRNAs for mouse Pdk1, Sgk1, and Sgk3 were purchased from Sigma. Viral particles were produced in HEK293T cells transfected with the plasmid of interest and appropriated packaging plasmids using jet Prime (Polyplus transfection). Target cells were infected with viral particles by spinoculation in the presence of polybrene (4 µg/mL; Sigma). Stable clones were established by growth in media containing puromycin (1 µg/mL; InVivoGen).

Western blotting
Cells were harvested and lysed in RIPA buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 µg/mL aprotinin, and 10 µg/mL leupeptin). Cell lysates were subjected to SDS-PAGE and the proteins were transferred onto nitrocellulose membranes (Osmonics Inc.). Membranes were incubated with primary antibodies for 18 hours at 4°C, washed, and then incubated with secondary antibody conjugated with fluorescent dye. After processing, membranes were analyzed using the Odyssey Imaging System (Amersham Biosciences).

Immunofluorescence microscopy
Sections of skin and lymph nodes prepared as described above were deparaffinized, rehydrated, washed in PBS, and incubated with Digo protein Block for 30 minutes at room temperature. Antibody retrieval for S100 immunostaining was performed by incubation in citrate buffer (pH 6.0) in a decloaking chamber (Biocare Medical). Antibodies were diluted in Dako antibody diluent at 1:500 and incubated with sections overnight at 4°C. Secondary antibodies conjugated to Alexa Fluor 543 (Molecular Probes) were diluted to 1:400 and incubated with sections for 1 hour at room temperature. Nuclei were counterstained with SlowFade Gold anti-fade reagent containing 4',6-diamidino-2-phenylindole (DAPI; Vector).

qRT-PCR analysis
Total RNA was extracted using a miniprep kit (Sigma) and digested with DNase I. cDNA was synthesized using oligo(dT) and random hexamer primers, and qPCR was performed on biological triplicates using SYBR Green. Amplification of histone H3.3A served as an internal control. The PCR primers were designed using Primer3 and their specificity was checked using BLAST. The PCR products were limited to 100 to 200 base pairs. Primer sequences were:

<table>
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Figure 1.
Loss of PDK1 inhibits the onset of melanoma development and delays metastasis. A and B, representative images of PDK1 WT (BrafV600E::Cdkn2a/-/-;Pten/-/-;Pdk1/-/+) or PDK1 KO (BrafV600E::Cdkn2a/-/-;Pten/-/-;Pdk1/-/-) mice 21 days after administration of 4-HT. C and D, representative images of the dorsal skin (C) and lymph nodes (D) from PDK1 WT and PDK1 KO mice 38 days after systemic administration of 4-HT. E, quantification of tumors in PDK1 WT and PDK1 KO mice (n = 12 and 8, respectively). F, Kaplan–Meier survival curves of PDK1 WT and PDK1 KO mice (n = 16 and 18, respectively). P < 0.001 by log-rank (Mantel-Cox) test. G and H, H&E-stained (G) and S100-immunostained (H) skin sections from PDK1 WT and PDK1 KO mice 38 days after 4-HT administration. Bars, 100 µm. J, I, Western blot analysis of the indicated proteins in primary melanoma cultures derived from PDK1 WT and PDK1 KO mice.
atccgtgtggacatacggtt-3'; mouse Tyr: forward, 5'-tcggacccagctggctttcc-3' and reverse, 5'-aactggcttcagggaaagt-3'; mouse il6: forward, 5'-actggacagctggccagag-3' and reverse, 5'-cctggaggagcagggaga-3'; mouse Mmp3: forward, 5'-gtcgggcttcagggaaagt-3' and reverse, 5'-catgggcttcagggaaagt-3'; mouse Zeb1: forward, 5'-acgccgtgattacg-3' and reverse, 5'-ctacgtgtggttcgatttctca-3'.

Quantification of lymph node metastasis

Immunofluorescent staining of S100 in lymph node sections was performed as described above. S100-positive tumor cells in the sections were quantified by first scanning at ×20 magnification with the Aperio ScanScope FL system (Aperio Technologies) and then analyzing cell numbers using the Aperio Quantification FL algorithm (version 11, Aperio Technologies). The algorithm was tuned using a preset procedure and the subsequent macro was saved and applied to all slides.

Colony formation assay

Five hundred tumor cells were plated into each well of a 6-well plate and incubated for 7 to 12 days. Viable colonies were stained with crystal violet (Sigma-Aldrich) and the plates were imaged. The colony numbers and intensity were determined using ImageJ software. Each experiment was performed at least 3 times.

Animal studies and in vivo experiments

All mouse experiments were performed under the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Sanford-Burnham Medical Research Institute. BrafV600E::Cdkn2a+/− and Pdk1+/− mice were generated as previously described (17, 18). Cohorts of at least 6 animals per group were used in each of the experimental groups.

Three-dimensional growth assay

Cells were induced to form spheroids using the hanging drop method. The cells were plated at 200 cells per 20 μL per well in a Nunc-60 well microwell MiniTray. The trays were covered, inverted, and incubated at 37°C in a humidified 5% CO2 incubator for 5 days. Spheroids from wells containing single spheroids were transferred to a 48-well plate coated with 1% low-melting-point agarose. Compounds or DMSO vehicle were added, and images of spheroids were captured every 48 hours for 8 days using an Olympus IX-71 microscope equipped with a camera. The relative spheroid areas were measured using ImageJ64 software.

Flow cytometric cell-cycle analysis

Cell lines were seeded in 6-well tissue culture plates at 1 × 10^5 cells per well, incubated overnight, and then treated with shRNAs. Cells were harvested by trypsinization, fixed in 70% ethanol in PBS at −20°C, and then stored until further use. For analysis, cells were washed once in PBS and incubated in cell-cycle staining buffer (60 μg/mL propidium iodide and 0.15 mg/mL RNase A; Sigma) for 20 minutes. Biological triplicates of 10,000 cells (within the G1 phase of the cell cycle; 82,000 nmol/L MgCl2, 1 mMol/L EGTA, 100 nmol/L NaF, 10 nmol/L NaPPi, 10% glycerol, 1 nmol/L PMSF, 1 nmol/L Na3VO4, aprotinin 10 μg/mL). Lysates were transferred at volumes of 25 to 30 μL into a PCR 96-well plate. Ten microliters of 4 × SDS/2-ME sample buffer (35% glycerol, 8% SDS, 0.25 mol/L Tris-HCl, pH 6.8; with 10% β-mercaptoethanol added before use) was added to each sample well. The plates were covered and incubated for 5 minutes at 95°C and then centrifuged for 1 minute at 2,000 rpm. Samples were applied to RPPA slides as previously described (19).

Reverse-phase protein array analysis

The cells were washed twice in ice-cold PBS, then lysed in 30 μL of reverse-phase protein array (RPPA) lysis buffer (1% Triton X-100, 50 nmol/L HEPES (pH 7.4), 150 nmol/L NaCl, 1.5 nmol/L MgCl2, 1 mmol/L EGTA, 100 nmol/L NaF, 10 nmol/L NaPPi, 10% glycerol, 1 nmol/L PMSF, 1 nmol/L Na3VO4, and 0.15 mg/mL RNase A, 1 nmol/L PMSF, aprotinin 10 μg/mL). Lysates were transferred at volumes of 25 to 30 μL into a PCR 96-well plate. Ten microliters of 4 × SDS/2-ME sample buffer (35% glycerol, 8% SDS, 0.25 mol/L Tris-HCl, pH 6.8; with 10% β-mercaptoethanol added before use) was added to each sample well. The plates were covered and incubated for 5 minutes at 95°C and then centrifuged for 1 minute at 2,000 rpm. Samples were applied to RPPA slides as previously described (19).

Synthetic lethal screen for PDK1 inhibitor combination

Interactions between PDK1 (GSK2334470) and 45 other agents were determined in a "one versus many" screen at the Yale Center for Molecular Discovery. A master 384-well plate was set up manually, incorporating 4 or 8 dilutions of 45 test agents to yield a range from 10 μmol/L to 1 nmol/L final in the experiments. The master plates also included multiple negative control wells (0.2% DMSO vehicle) and staurosporine-positive "kill" controls for a final 10 μmol/L staurosporine. The test agents consisted of 2,4-dinitrophenol, 4u8c, 17-DMAG, 4485, atorvastatin, AZD-2014, AZD-6244, BI-D1870, BMK-120, bortezomib, brefeldin A, carfilzomib, cerulenin, EMD63863, EX 527, FASN21l, fatostatin A, FCCP, GGTI-298, GSK650394A, GSK690693, GSK2343470, GSK2606416, homoharringtonine, metformin, oligomycin, PF429242, phenformin, piperlongumine, rosiglitazone, SB 405940, SBR1108610, SBR1108634, SBR1108684, SBR1108692, SBR1108734, SBR1108740, simvastatin, SRT1720, STA-4783, STF-20083010, T0070907, trametinib, tunicamycin, and vemurafenib. A total of 750 to 1,000 cells per 16 μL medium per well were dispensed into 384-well plates using a Thermo multidrop dispenser. After incubation overnight to allow cell attachment, a pin tool was used to transfer 20 nL of compounds from each well of the master plate into the 384-well test plates containing cells. Next, 4 μL of either 0.1% DMSO or GSK233470 to yield final 2 or 10 μmol/L were added. Cells were incubated an additional 3 days, and growth was quantified by CellTiterGlo (Promega) to read out ATP accumulation, which correlates well with viable cell number. Additivity and superadditivity were calculated using the Bliss independence model and area under the curve (AIC) calculations. To avoid overestimating the high concentration points, the natural log concentrations are used to calculate AIC. AIC calculation is performed using the function "auc" from the R library "MESS," using the linear type option. This function approximates the area under a set of points by summing the trapezoidal segments between adjacent points. Normalization of the AIC calculation is performed by dividing the calculated AIC by the width of the log concentration range for each plated agent to correct for differences in concentration ranges.

Statistical analysis

All data except survival curves were analyzed by the unpaired t test. Kaplan–Meier survival curves were compiled using GraphPad software (GraphPad) and statistical significance was assessed using the log-rank (Mantel–Cox) test. P < 0.05 was considered statistically significant.

Results

PDK1 deletion promotes tumor suppression and reduces the pigmentation of BrafV600E::Cdkn2a+/− mice harboring WT Pten

Our previous studies demonstrated that the genetic inactivation of Pdk1 in BrafV600E::Cdkn2a+/−:Pten+/− animals significantly delays melanoma development and effectively inhibits...
Figure 2.

shRNA-mediated inhibition of PDK1 or SGK3 suppresses phosphorylation of target proteins, expression of cyclin D1, and growth of melanoma cells. A, Western blot analysis of the indicated proteins in primary melanoma cultures derived from Braf^{V600E::Cdkn2a/C0/C0::PTEN/C0/C0} mice (YUMM1.5 and YUMM1.9) or Braf^{V600E::Cdkn2a/C0/C0::PTEN^{+/+}} mice (SBM-A2 and SBM-A3). B, Western blot analysis of the indicated proteins from YUMM1.5 and SBM-A2 melanoma cells expressing control, Sgk1-, Pdk1-, or Sgk3-targeted shRNA. C and D, colony formation assay of SBM-A2 (C) and YUMM1.5 (D) cultures expressing the indicated shRNAs. Individual wells shown are representative of three experiments with triplicate cultures. The graph represents the quantification of colony-forming ability compared with control shRNA. Error bars, SEM. E, representative images of YUMM1.5 spheroids expressing the indicated shRNAs. Relative spheroid sizes were quantified using ImageJ software (NIH) and are presented as the mean ± SEM of ≥6 spheroids per group.
metastasis (5). Because about 70% of human melanomas harbor WT PTEN, we sought to determine the effects of Pdk1 deletion on the development and progression of melanoma in BrafV600E::Cdkn2a−/−::Pten+/+ mice. In these mice, the expression of BrafV600E is induced specifically in melanocytes by the administration of the estrogen analog 4-HT (5).

Figure 3.
Pharmacologic inhibition of PDK1 or SGK3 inhibits target protein phosphorylation and melanoma cell growth. A, Western blot analysis of the indicated proteins in YUMM1.5, YUMM1.9, SBM-A2, and SBM-A3 cells treated for 24 hours with DMSO (vehicle), 10 μmol/L GSK2334470 (PDK1i), or 10 μmol/L GSK650394 (SGKi). B and C, spheroid formation by YUMM1.5 (B) and YUMM1.9 (C) cells incubated with 5 or 20 μmol/L inhibitors. Spheroid volumes were quantified on the indicated days as described for Fig. 2. Results are the mean ± SEM of ≥6 spheroids per group.
Pdk1−/− (PDK1 KO) mice systemically treated with 4-HT on postnatal days 1, 3, and 5 exhibited markedly reduced pigmentation on day 21 after 4-HT administration compared with similarly treated BravO600E::Cdkn2a−/−::Pdk1+/+ (Pdk1 WT) mice (Fig. 1A and B). Consistent with these observations, heavy pigmentation and melanomagenesis was observed in the skin (Fig. 1C) and lymph nodes (Fig. 1D) of PDK1 WT mice whereas it was significantly reduced in the PDK1 KO mice. Examination of mice on day 38 after 4-HT administration revealed that 7 of 12 (40%) PDK1 WT mice contained tumors, whereas tumors were undetectable in the PDK1 KO mice (Fig. 1E). Moreover, the mean survival time of the PDK1 KO mice was significantly prolonged compared with that of the PDK1 WT mice (76 vs. 42 days; \( P < 0.001 \); Fig. 1F). Notably, only a single administration of 4-HT was required to observe the effects of PDK1 deletion on melanomagenesis in the BravO600E::Cdkn2a−/−::Pdk1−/− mice, which enabled the analysis of AGC kinase pathway components. As expected, the absence of PDK1 resulted in a marked reduction in the phosphorylation of downstream target proteins such as AKT, FOXO3a, GSK3β, PRAS40, RSK, p70S6K, and the SGK1 substrate NDRG1 (Fig. 1K).

Collectively, these data establish that PDK1 plays an important role in the development of melanoma in BravO600E/WT Pten, in addition to its reported role in the BravO600E mice in which Pten is inactivated or deleted.

SGK1 and SGK3 are key mediators of PDK1-dependent melanomagenesis

The results shown above identify several potential PDK1 substrates that might be required for melanoma development in Pdk1 WT mice, including the transcription factor FOXO3a, which was also identified in our earlier study (5). Notably, FOXO3a is regulated by phosphorylation of the same phosphoacceptor sites by either AKT or SGK. To determine whether SGK1 or SGK3 plays a role in PDK1-dependent melanoma development, we performed shRNA-mediated knockdown (KD) of Pdk1, Sgk1, or Sgk3 in two melanoma lines each derived from BravO600E::Cdkn2a−/−::Pten−/−/+ mice (lines YUMM1.5 and YUMM1.9) and BravO600E::Cdkn2a−/−::Pten−/−/+ mice (lines SBM-A2 and SBM-A3) and examined the effects of KD on the expression and phosphorylation of key PDK1 signaling components (Fig. 2A and Supplementary Fig. S2A–S2D). As expected, the degree of AKT, FOXO3a, and PRAS40 phosphorylation was notably lower in the PTEN WT tumor cells, reflecting PTEN activity (Fig. 2A). The degree of the
KD for Pdk1, Sgk1 and Sgk3 was largely efficient (Supplementary Fig. S2A–S2D). PDK1 KD effectively attenuated phosphorylation of FOXO3a, NDRG1, S6, P70S6K, and 4EBP1 in both 

**Pharmacologic inhibition of SGK3 inhibits melanoma cell growth**

We next asked whether pharmacologic inhibition of PDK1 and SGK would phenocopy the genetic inactivation with shRNA. To this end, we treated the 

**Figure 5.** Combination screen of PDK1i against 45 candidate test agents. A, heat map of model-free AUC of GSK2334470 at each of the two concentrations tested with unsupervised clustering of test agents (rows) and cell lines (columns). Red dots mark bortezomib, carfilzomib, and BKM-244 combinations with 10 μmol/L GSK2334470. Dilutions of test agents were combined with either 0.1% DMSO vehicle, 2 μmol/L GSK2334470, or 10 μmol/L GSK2334470 and incubated with cell lines and assayed after 3 days with CellTiterGlo as described in Materials and Methods. Cell lines tested were YURO8, YUHEF (WT BRAF, NRAS), YUMAC, YUSIK (BRAF), YUGASP (NRAS). B, Western blot analysis of the indicated proteins in mouse melanoma cells SBM-A2 and YUMM1.9 and human melanoma cell lines UACC903 and Mel501 treated for 24 hours with 0.1, 1, or 5 μmol/L of GSK2334470 (PDK3h) in the presence or absence of the protesome inhibitor bortezomib (BTZ) at 1 nmol/L (SBM-A2) or 4 nmol/L (YUMM1.9, UACC903, Mel501), C, growth of YUMM1.5 spheroids treated with the indicated concentrations of GSK2334470 (PDK3i) and bortezomib alone or in combination. Relative areas were calculated as described for Fig. 2. Values are the mean ± SEM of ≥6 spheroids per group.

**Inhibition of SGK3 or PDK1 causes melanoma cell-cycle arrest at G1**

To determine the mechanism(s) by which genetic or pharmacological inhibition of SGK3 suppresses melanoma growth, we performed FACS analysis to determine the proportion of cells in each phase of the cell cycle after KD of PDK1 or SGK3. We found that the expression of either shSgk3 or shPdk1 led to the accumulation of 

**Combination therapies synergize with proteasome and PI3K/mTOR inhibition to attenuate melanoma growth**

Combination therapies are often more potent inhibitors of tumor growth and can also suppress the growth of tumors resistant to the individual therapies. Therefore, we next screened for pharmacologic inhibitors that might act synergistically with PDK1i in suppressing melanoma growth. For this, we tested the 

**Summary and conclusions**

In summary, we have shown that SGK3 and PDK1 play a critical role in melanoma growth and survival through multiple mechanisms involving the PI3K/AKT/mTOR signaling pathway. Our findings provide a rationale for the development of new therapeutic strategies targeting these proteins in melanoma treatment.
and bortezomib was observed with the human melanoma cell lines UACC903 and Mel501, although the degree of caspase-3 cleavage was not detectable in the UACC903 cells, possibly due to the higher level of AKT in these cells (Fig. 5B). Notably, the synergistic effect on apoptosis of YUMM1.5 cells following cotreatment of bortezomib and PDK1 inhibitors was seen in 3D cultures only when higher concentrations of bortezomib were administered (200 nmol/L in 3D cultures, compared with 4 nmol/L in the 2D cultures; Fig. 5C). The latter might be attributed to the higher expression of antiapoptotic genes in cells grown in 3D cultures, as previously reported for lung cancer cells and mesothelioma cell lines (23, 24).

The pan-PI3K inhibitor BKM120 (25, 26) was also found to synergize with the PDK1 inhibitor BKM120 (25, 26) was also found to synergize with the PDK1 inhibitor BKM120 in reducing AKT, FOXO3a, PRAS40, and GSK3β phosphorylation in both the PTEN WT and mutant melanoma cells (Fig. 6A). Notably, the combination of BKM120 with PDK1 induced cell death in the PTEN WT but not in the PTEN-mutant melanoma cells, measured by cleaved caspase-3 levels (Fig. 6A). Nevertheless, the combination of BKM120 and PDK1 attenuated cell growth in the PTEN-mutant melanoma cells, as reflected by the reduced expression of cyclin D1 (Fig. 6A). These observations indicated that the response of melanoma cells to combined inhibition of PI3K/PDK1 depends on the degree of PTEN/AKT signaling and determines the susceptibility to undergo cytostatic or cytotoxic response.

We have further assessed the effect of the PDKi and BKM120 combination on human melanoma harboring PTEN WT or mutant genotypes. Consistent with the findings in the mouse melanoma lines, this combination was effective in inhibiting the key AKT/PDK1 signaling in both melanoma lines, albeit with greater effectiveness on the PTEN WT–derived cells. Cell death program, monitored by caspase-3 cleavage, was induced in the PTEN WT (SBM-A2 and Mel501) but not PTEN-mutant (YUMM1.5 and UACC903) melanomas by the BKM120 and PDK1 inhibitor combination (Fig. 6B). When assessed in 3D growth, this combination elicited cytostatic effect, limiting, albeit significantly, the growth of these tumors (Fig. 6C).

The combined effect of PDK1 and PI3K inhibitors on the human melanoma cell lines UACC903 was analyzed by RPPA using a panel of 172 antibodies directed to components of major signaling pathways (19, 27). This analysis revealed that combined PDKI and PI3K inhibition elicited an additive effect, which was reflected in the degree of decreased phosphorylation of key components along the PDK1 pathway, including S6, AKT, p70S6K, as well as tuberin, mTOR, and GSK3β, pointing to effect of this combination also on mTOR and GSK3 pathways (Supplementary Table S1, Fig. 5A, and Supplementary Fig. S5).

These findings points for the effectiveness of combined PI3K/PDK1 inhibition, which attenuates the growth of melanoma cells, with a notably greater effect on the PTEN WT cultures.

**Stratification of melanoma to PDK1 inhibition**

To determine whether sensitivity of melanoma to PDK1 is associated with a specific gene signature, we assessed the effects of PDK1i on 19 melanoma cell lines for which gene expression and genomic mutation data are available. The PDK1i 50% inhibitory concentration (IC50) for cell growth and 50% effective concentration (EC50) were used to segregate the cell lines (Fig. 7A). The seven most resistant (UACC9140, 2994, 2512, 1120, 1118, 558, and 264I) and the seven most sensitive (UACC903, 2331, 612, 2496, 647, 952, and 3337) cell lines were then selected for Ingenuity Pathway Analysis (IPA) to identify the genes and pathways most significantly affected by PDK1i (P < 0.05). This analysis identified 1,178 differentially expressed genes, including multiple components of the TIF2 nuclear co-regulator and mTOR signaling pathways that were specifically associated with sensitivity to PDK1i. Further refinement of this assessment to include only genes showing ≥10-fold differential expression clearly distinguished 2 clusters of genes associated with sensitivity and resistance to PDK1i (Fig. 7B). Sensitivity to PDK1i was associated with the reduced expression of several pigmentation genes (DCT, PMEL, MelanA) and elevation of number of cytokines and immunomodulators, including IL8, IL1B, IL6, Serpin1, Serpin2, and PDGF (Fig. 7B). These findings were confirmed by qPCR. PDK1i-treated YUMM1.5 cells showed a reduction in DCT and TYR mRNA and an increase in IL6 mRNA compared with untreated cells (Fig. 7C). Consistent with these results, canonical pathway analysis showed that sensitivity of melanoma cells to PDK1i was associated with decreased expression of MITF, the key upstream regulator of pigmentation genes (P = 1.6E-16). Integrating signaling genes were also strongly associated with PDK1i sensitivity (P = 7.1E-6). Analysis of mRNA levels confirmed reduced expression of Tyr and Dct. 2 pigmentation genes in both PTEN WT and mutant melanoma cells derived from the Pdk1 KD tumors (Supplementary Fig. S6). Likewise, lack of Pdk1 in these melanomas also attenuated the expression of epithelial–mesenchymal transition (EMT)-related genes (28), Zeb1 and Mmp3 (Supplementary Fig. S6). Analysis of 3 melanoma lines revealed slight differences (i.e., effect on Tyr level in YUMM1.9 and on MMP3 in YUMM1.5 cells was limited, compared with changes seen in the other 2 cultures), pointing to heterogeneity among the cell types used here. These findings points to a number of genes that could be further explored as markers for the stratification of patients with melanoma for therapy by PDK1 inhibition.

**Discussion**

A number of pathways are known to contribute to melanoma development and progression, including the MAPK signaling pathway, which is deregulated in more than 70% of melanomas (NRAS and BRAF mutations), and the PI3K/AKT signaling pathway, which is deregulated in more than 50% of these tumors, in part due to genetic mutations and in part due to altered posttranslational modifications (29, 30). Using genetic and pharmacologic inhibitors, we previously demonstrated the importance

Figure 6. PDK1 and PI3K/mTOR inhibitors synergize to suppress melanoma cell growth. A, Western blot analysis of the indicated proteins in YUMM1.9 and SBM-A2 cells treated for 24 hours with 0.1, 1, or 5 μmol/L of GSK2334470 (PDKi) in the presence or absence of 1 or 3 μmol/L of the dual PI3K/mTOR inhibitor BKM120. B, Western blot analysis of the indicated proteins in the human melanoma cell lines UACC903 and Mel501 treated with 0.1, 1, or 5 μmol/L of GSK2334470 (PDKi) in the presence or absence of 1 or 3 μmol/L of BKM120. C, growth of YUMM1.5 spheroids treated with the indicated concentrations of GSK2334470 (PDKi) and BKM120 alone or in combination. Relative areas were calculated as described for Fig. 2. Values are the mean ± SEM of ≥6 spheroids per group.
of the master AGC kinase—PDK1—for the development and metastasis of BrafV600E Pten−/− melanomas (5). Here, we extend those findings to show that PDK1 also plays an important role in melanomas harboring WT PTEN, which includes about 70% of human melanomas. Significantly, we found that PDK1 actually plays a more pronounced role in PTEN WT than PTEN-mutant genotypes, a finding that is expected because of the preserved PTEN activity, which harnesses AKT and related PI3K

Figure 7.
Stratification of human melanomas by sensitivity to PDK1 inhibition. A, the mean IC50 for GSK2334470 inhibition of growth of 19 human melanoma cell lines was used to segregate cell lines into sensitivity or resistance to the inhibitor. B, heat map of the results of the IPA showing the genes/pathways most significantly altered (P < 0.05; fold change > 1.5) by GSK2334470 (1,178 differentially expressed genes). C, qPCR analysis of Dct, Tyr, and Il6 mRNA in YUMM1.5 cells treated with DMSO or GSK2334470 at 5 μmol/L for 24 hours (Dct and Tyr) or at 10 μmol/L for 6 hours (Il6). Values are the mean ± SEM of biologic triplicates. *, P < 0.05; **, P < 0.01.
components. Together, our findings further our appreciation for the impact of PDK1 to the AGC kinase landscape.

The significance of our observations is highlighted in synthetic lethal screens by the identification of pan-PISK inhibitor that effectively synergize with PDK1, particularly in PTEN WT melanomas, suggesting that the focused targeting of the PI3K–PDK1 signaling axis might represent a novel therapeutic modality for PTEN WT melanomas. A similar approach with combination MK6 and BRAFi has produced promising results in ongoing clinical trials (31). Notably, our data point to a gene signature that distinguishes between PDK1- sensitive melanomas (which express low levels of pigmentation genes associated with MITF signaling) and PDK1-resistant melanomas (which express inflammation-related genes, including Il6 and Il3); this signature could potentially be used for the stratification of patients for PDK1-targeted therapies. Given the recent finding that low MITF expression correlates with resistance in melanoma, colon, and thyroid cancer cells (33), it would be of interest to further examine the possibility that targeting the PDK1 pathway might also be efficient for inhibiting the resistance phenotype. Notably, PDK1-deficient melanomas also exhibited reduced ZEB1 and MMP3 expression, pointing to PDK1 role in control of the EMT, elucidating the reduced metastasis observed in vivo and attenuating growth in 3D in culture. Of interest, our results reveal that inhibition of either PDK1 or SGK3 decreased the phosphorylation of 4-EBP1, pointing to possible roles of AGC kinases in the regulation of CAP-dependent translation. Consistent with these observations, eIF4E, another component of this translational initiation complex, was recently linked with the resistance to anti-BRAF and anti-MEK therapies in BRAF-mutant melanoma, colon, and thyroid cancer cells (33).

PDK1 inhibitors that are suitable for use in clinical trials have not yet been developed. Although the PDK1i used in our study (GSK2334470) is quite specific and exhibits excellent properties for work with cultured cells (22, 34), it is unsuitable for further preclinical or clinical development. Other PDK1 inhibitors are currently being developed, which might exhibit acceptable safety profiles and be eligible for further advanced clinical evaluation.

An alternative approach to inhibiting PDK1 is to identify and target one or more downstream PDK1 substrates that are crucial for melanoma development and progression. In this regard, we report here that the genetic and pharmacologic inhibition of SGK3 largely phenocopies the effects of PDK1 inhibition on melanoma cells, including growth arrest in G1 phase. Our findings are consistent with earlier reports that pointed to the role of SGK3 in melanoma (8).

The repertoire of potential targets for melanoma therapy is expanding considerably, thanks in large part to the extensive knowledge gained from mechanistic and clinical studies. As a result, future clinical management options will be more extensive and are likely to include modulators of pathways that are independent of the MAPK signaling axis. Our data substantiate the importance of PDK1 and one of its downstream substrates, SGK3, for melanoma development and progression and further suggest that inhibitors of this signaling pathway might be useful for the development of clinical management options for melanoma.

Disclosure of Potential Conflicts of Interest

M.A. Davies reports receiving commercial research grants from GlaxoSmithKline, AstraZeneca, Merck, Genentech, Myriad, Sanofi-Aventis, and Oncobody and is a consultant/advisory board member for GlaxoSmithKline, Novartis, Genentech, and Sanofi-Aventis. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: M. Scortegagna, H. Yin, D.F. Stern, M. Bosenberg, Z.A. Ronai
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Scortegagna, E. Lau, C. Seredu, C.G. Langdon, R. Halaban, M. Pellecchia, M. Bosenberg
Writing, review, and/or revision of the manuscript: M. Scortegagna, E. Lau, T. Zhang, H. Yin, M.A. Davies, D.F. Stern, M. Bosenberg, Z.A. Ronai
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Scortegagna, T. Zhang, K. Mereth, R. Halaban, Z.A. Ronai
Study supervision: M. Scortegagna, Z.A. Ronai
Other (synthesis of the compound): S.K. De

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PDK1 and SGK3 Contribute to the Growth of BRAF-Mutant Melanomas and Are Potential Therapeutic Targets

Marzia Scortegagna, Eric Lau, Tongwu Zhang, et al.

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