**AKT1 Activation Promotes Development of Melanoma Metastases**

**Highlights**

- Expression of activated AKT1 promotes melanoma formation and metastasis

- Loss of *Pten* cooperates with AKT1 activation to promote melanoma metastasis

- mTOR signaling downstream of AKT1 is implicated in driving metastasis

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**In Brief**

AKT signaling has been implicated in melanoma metastasis; however, the ability of activated AKT1 to promote melanoma metastasis in vivo has not been explored. Cho et al. show that expression of activated AKT1 in BRAF^{V600E}/Cdkn2a^{Null} melanomas is sufficient to promote tumor cell dissemination to the lungs and brain.
AKT1 Activation Promotes Development of Melanoma Metastases

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INTRODUCTION

Recent approvals of more efficacious therapies have significantly shifted the treatment paradigm for melanoma and have provided much needed breakthroughs in this disease (Girotti et al., 2014). Despite these recent therapeutic advances, the majority of melanoma deaths continue to be due to metastasis, which demands further investigation into the molecular mechanisms driving distant dissemination of this disease.

Members of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway have been implicated in melanoma initiation, progression, invasion, and metastasis. Activation of AKT in this disease occurs most commonly through silencing of the tumor suppressor PTEN (reviewed in Madhunapantula and Robertson, 2011). PTEN is a phosphatase with activity against both lipid and protein substrates (Chalhoub and Baker, 2009). Its major substrate is phosphatidylinositol-3,4,5-triphosphate (PIP3), which recruits AKT to the membrane, where it is activated by phosphorylation. PTEN specifically dephosphorylates the 3’ position of PIP3 to create phosphatidylinositol-4,5-bisphosphate (PIP2) and thereby suppresses membrane recruitment and downstream signaling of AKT. Loss of PTEN results in increased levels of PIP3 and subsequent AKT activation.

We have previously demonstrated that PTEN silencing combined with expression of BRAFV600E in mouse melanocytes in vivo results in melanoma formation (Dankort et al., 2009). Our current study builds on these findings and demonstrates that ectopic expression of activated AKT1 strongly potentiates metastasis in the context of mutant BRAFV600E and silencing of INK4A and ARF. Additionally, PTEN silencing cooperates with active AKT1 to accelerate both tumor formation and metastasis. This model provides a valuable tool to further define the mechanisms that promote melanoma metastases and a powerful platform to advance the development of anti-melanoma therapies.

RESULTS

BRAFV600E/Cdkn2aNull Melanomas Are Not Metastatic

To evaluate melanoma metastasis in the context of specific genetic alterations, we utilized an established melanoma mouse model based on the RCAS/TVA system that allows for targeted delivery of specific genes to post-natal melanocytes (VanBrocklin et al., 2010). This system utilizes a viral vector, RCASBP(A), hereafter referred to as RCAS, and its receptor, TVA. Transgenic mice that express TVA under the control of the dopachrome tautomerase (DCT) promoter allow targeting of the virus, and expression of the genes it contains, specifically to melanocytes. To assess metastasis using this model in the context of mutational activation of BRAFV600E, we compound generated Dct::TVA;BRafCA;Cdkn2alox/lox mice (VanBrocklin et al., 2010) carrying a conditional Cre-activated allele of Braf.
The Braf<sup>CA</sup> allele expresses wild-type BRAF prior to Cre-mediated recombination after which BRAF<sup>V600E</sup> is expressed from the normal chromosomal locus (Dankort et al., 2007). The Cdkn2α<sup>lox/lox</sup> allele expresses normal INK4A and ARF prior to Cre-mediated recombination after which expression of both p16<sup>INK4A</sup> and p19<sup>ARF</sup> is extinguished (Aguirre et al., 2003).

Newborn Dct::TVA;Braf<sup>CA</sup>;Cdkn2α<sup>lox/lox</sup> mice were injected subcutaneously with an RCAS virus encoding Cre to induce BRAF<sup>V600E</sup> expression with concomitant silencing of INK4A and ARF in melanocytes. While Braf<sup>CA</sup>;Cdkn2α<sup>lox/lox</sup> mice lacking Dct::TVA injected with RCAS:Cre remained tumor free for the duration of the study (150 days, n = 40), 47% (16/34) of the Dct::TVA;Braf<sup>CA</sup>;Cdkn2α<sup>lox/lox</sup> mice infected with the RCAS:Cre virus developed tumors at the site of injection (Table S1). The mean survival for tumor-bearing mice in this cohort was 88.9 ± 8.6 days (Figure 1A). All major organs were examined at euthanasia, but no melanoma metastases were observed in any of the tumor-bearing mice.

**PTEN Silencing Increases Tumor Incidence and Reduces Tumor Latency but Does Not Significantly Enhance Metastasis to Distant Organs**

Because BRAF<sup>V600E</sup> cooperates with PTEN silencing to induce metastatic melanoma (Dankort et al., 2009), we generated Dct::TVA;Braf<sup>CA</sup>;Cdkn2α<sup>lox/lox</sup>;Pten<sup>lox/lox</sup> mice. The Pten<sup>lox</sup> allele used here expresses normal PTEN prior to Cre-mediated recombination after which deletion of the exon 5 sequence generates a Pten null allele (Zheng et al., 2008). Newborn Dct::TVA;Braf<sup>CA</sup>;Cdkn2α<sup>lox/lox</sup>;Pten<sup>lox/lox</sup> mice were injected subcutaneously with RCAS:Cre to induce BRAF<sup>V600E</sup> with concomitant silencing of INK4A, ARF, and PTEN in melanocytes. Importantly, control mice, Braf<sup>CA</sup>;Cdkn2α<sup>lox/lox</sup>;Pten<sup>lox/lox</sup> mice injected with RCAS:Cre lacking Dct::TVA infected with RCAS:Cre remained tumor free for the duration of the study (150 days, n = 40). Tumors developed at the site of injection in all of the Dct::TVA-positive Braf<sup>CA</sup>;Cdkn2α<sup>lox/lox</sup>;Pten<sup>lox/lox</sup> mice infected with RCAS:Cre viruses (n = 24). The mean survival was 57.8 ± 3.4 days in this cohort (Figure 1A; Table S1). Expression of Cre was assessed by RT-PCR (Figure S1A), and recombination of Pten<sup>lox</sup> was confirmed by PCR in all of the tumors that developed (Figure S1B). In these mice, lung metastases were detected in 8.3% (2/24) of the mice whose melanomas had PTEN silencing. However, using a Fisher’s exact test, we determined that this difference was not statistically significant when compared with mice whose tumors expressed PTEN (p = 0.5).

**Expression of Activated AKT1 Promotes Melanoma Formation and Distant Metastasis**

Several studies have shown that PTEN silencing confers a different phenotype than AKT activation (Majumder et al., 2003; Wang et al., 2003). Therefore, we evaluated the effect of...
mutationally activated AKT1 on melanoma formation and progression in vivo. Newborn Dct::TVA;BrafCA;Cdkn2alox/lox (PtenWT) mice were injected subcutaneously with RCAS viruses encoding myristoylated (myr) Akt1 alone or in combination with viruses encoding Cre. Virally delivered myrAkt1 contains a hemagglutinin (HA) epitope tag to discern expression from endogenous AKT1. While Dct::TVA;BrafCA;Cdkn2alox/lox mice injected with viruses encoding myrAkt1 alone remained tumor free for the duration of the study (150 days, n = 21), 88% (24/27) of the mice injected with both myrAkt1- and Cre-encoding viruses developed tumors at the site of injection (Figure 1A; Table S1). The mean survival of the tumor-bearing mice in this cohort was 65.3 ± 4.7 days (Figure 1A). It is important to note that AKT1 expression is not required for tumor formation in the context of BRAFV600E/INK4A-ARF silencing; tumors develop in nearly half of Dct::TVA;BrafCA;Cdkn2alox/lox mice injected with viruses encoding Cre only (Figure 1A). Of the 24 mice that developed tumors when injected with myrAkt1 and Cre viruses, AKT1 expression was detected in 18 of the tumors by IHC for the HA epitope tag on myrAkt1 (Figure S2). The mean survival was 58.9 ± 3.5 in this myrAkt1-confirmed cohort (Figure 1B; Table S1). A significant difference in survival was observed between mice whose tumors expressed myrAkt1 and those without myrAkt1 (p = 2.3 × 10−3). A significant difference in survival was also observed between the Ptenlox/lox cohort injected with viruses encoding Cre only when compared with all of the mice in the PtenWT cohort injected with two separate viruses encoding Cre and myrAkt1 (p = 0.045) (Figure 1A; Table S1). Comparison between the mice whose tumors were found to express myrAkt1 by immunohistochemistry (IHC) (18/24) and mice whose tumors lacked PTEN revealed no significant difference in survival (p = 0.994; Figure 1B). All major organs were examined in tumor-bearing mice. Interestingly, lung metastases were observed in 67% (12/18) and brain metastases were observed in 17% (3/18) of the mice whose tumors expressed myrAkt1. This difference was statistically significant when compared with both the PtenWT and the Ptenlox/lox cohorts injected with RCAS:Cre viruses (p < 0.0001).

PTEN Silencing Cooperates with Activated AKT1 to Accelerate Melanomagenesis and to Promote Distant Metastasis

Our data revealed differences in the development of distant metastases between cohorts of mice whose tumors lacked PTEN compared with mice whose tumors expressed myrAkt1. Therefore, we assessed whether PTEN silencing could cooperate with myrAkt1 activation to promote melanoma formation and progression. To test this, newborn Dct::TVA;BrafCA;Cdkn2alox/lox, Ptenlox/lox mice were injected subcutaneously with viruses encoding myrAkt1 and Cre. Tumors developed at the site of injection in all of the mice in this cohort (n = 14), and myrAkt1 expression was detected in all of these tumors by anti-HA IHC (Figure S3). Expression of Cre was assessed by RT-PCR (Figure S1C), and recombination of Pten was confirmed by PCR in all of the tumors that developed (Figure S1D). Interestingly, melanomas with concomitant PTEN silencing and myrAkt1 expression had significantly reduced survival compared with mice whose tumors only had PTEN silencing (p = 0.0009) or expressed myrAkt1 in the presence of PTEN (p = 0.0002). The mean survival in this cohort was 42.1 ± 2.8 days (Figure 1B; Table S1). All major organs were examined, and lung and brain metastases were observed in 71% (10/14) and 79% (11/14) of the mice, respectively. A comparison of the sites of metastases revealed no significant difference in lung metastases (p = 1.0), but there was a statistically significant difference in brain metastases between tumors driven by myrAkt1 in the presence or absence of PTEN (p = 0.0009).

Histological Characterization of the Mouse Melanomas Reveals Features Similar to the Human Disease

Melanomas arising in Dct::TVA;BrafCA;Cdkn2alox/lox mice infected with both RCAS:Cre and RCAS:myrAkt1 encoding viruses consisted primarily of short spindle cells, occasional epithelioid cells, and with high-grade nuclear features, including prominent nucleoli. Intratumoral hemorrhage, coagulative tumor necrosis, and non-brisk inflammation (tumor infiltrating lymphocytes) were variably noted (Figure 2A). Mitotic figures were abundant and the majority of the cells expressed the Ki67 proliferation marker (Figure 2B). The activity of BRAFV600E was assessed by IHC for pERK as a surrogate (Figure 2C). The melanocytic origin of these tumors was established by their immunoreactivity for a pan-melanoma cocktail consisting of antibodies to HMB-45, a pre-melanosomal glycoprotein gp100, and melanoma antigen recognized by T cells-1 ( MART-1) (Figure 2D). The combination of positivity for both HMB-45 and MART-1 is highly supportive of a melanoma diagnosis (Ohsie et al., 2008). Upon gross examination, lesions were visible on the surface of the lungs (Figure 2E) and microscopic examination confirmed the morphologic features of malignancy (Figures 2F and 2G). AKT1 expression was detected in the metastases by IHC for the HA epitope tag on myrAkt1 (Figure 2H). Brain metastases were also observed in Dct::TVA;BrafCA;Cdkn2alox/lox mice infected with both Cre and myrAkt1 containing viruses (Figure 2I). Expression of myrAkt1 was confirmed in the brain metastases by IHC for the HA tag on virally delivered myrAkt1 (Figure 2J).

Primary tumors from each of the cohorts described above were assessed for the presence of phosphorylated AKT (pS473) by IHC and compared with BRAFV600E/INK4A-ARFnull melanomas (Figures 3A and 3B). As expected, active pS473-AKT was detected in tumors lacking PTEN, expressing myrAkt1, or both (Figures 3C–3H). The levels of pS473-AKT were also assessed by immunoblot analysis of lysates generated from fragments of tumors with wild-type PTEN, lacking PTEN, expressing myrAkt1, or lacking PTEN and expressing myrAkt1 (Figure 4A). In agreement with the IHC data, PTENnull melanomas displayed higher levels of pS473-AKT (Figure 4B). Expression of myrAkt1 was confirmed by immunoblotting for the HA epitope tag on myrAkt1 (Figure 4A) and quantified (Figure 4B). Cre-mediated expression of mutationally activated BRAF was assessed using an antibody specific for BRAFV600E. Activity of the MAPK pathway was assessed by analysis of phosphorylated ERK expression. Total levels of ERK were similar between the samples. As expected, the levels of total AKT were higher in the melanomas engineered to express myrAkt1 and a wider band representing the larger molecular mass of myrAkt1 was detected on the total AKT blot (Figure 4A).
Reverse-Phase Protein Array Analysis Reveals Increased mTOR Signaling in Tumors Expressing myrAKT1

To define differences in signaling between the melanomas lacking PTEN and those expressing myrAKT1 in a high-throughput manner we used a reverse-phase protein array (RPPA) approach. RPPA allows quantitative analysis of protein levels and activation using small amounts of protein (Liotta et al., 2003). Tumor-enriched protein isolates from five PTENNull and four myrAKT1-expressing melanomas were analyzed by RPPA. The heatmap in Figure 5 shows the results of unsupervised hierarchical clustering of the results of this analysis, which showed a
significant difference in protein modification/expression levels (n = 11; p < 0.05 by unpaired t testing) between the five PTENNull tumor samples and the four myrAKT1-expressing melanoma samples. A list of all 131 epitopes assessed with their respective p values is presented in Table S2. As expected, lower levels of PTEN expression were detected in the melanoma samples from Ptenlox/lox mice. In agreement with both the IHC and immunoblot analyses, a higher level of pS473-AKT was observed in the PTENNull tumor samples. Interestingly, components of the mammalian target of rapamycin (mTOR) signaling pathway (e.g., RAPTOR, RICTOR, NF2, and MYH11) were significantly different between the two groups (Figure 5; Figure S4).

**Pharmacological Inhibition of PI3K and mTOR Reduces Melanoma Cell Migration**

Pharmacological inhibition of PI3K and mTOR has been shown to inhibit melanoma cell growth both in vitro and in vivo and interfere with angiogenesis (reviewed in Sznol et al., 2013). To determine the dependence of melanoma cell migration on PI3K/

mTOR signaling, we treated normal human embryonic melanocytes (NHEM) and human melanoma cell lines A375, M14-MEL, and CACL, which harbor mutant BRAF, with DMSO or the pharmacological inhibitor NVP-BEZ-235 (BEZ-235), a dual PI3K/mTOR inhibitor. While very little migration was observed for NHEM, all three melanoma cell lines were highly migratory in this assay. Treatment with BEZ-235 (2 μmol/l) significantly reduced migration in the NHEM and all three melanoma cell lines over a 48-hr time period compared with DMSO treatment alone (Figure S5A). To examine the effects of BEZ-235 on signal pathway activity, extracts of melanoma cells treated with BEZ-235 (2 μmol/l) for 24 hr were subjected to immunoblot analysis (Figure S5B). BEZ-235 elicited a 96% reduction in phosphorylated AKT (pAKT; pS473) in A375 and M14 melanoma cells and a 90% reduction in phosphorylated AKT in CACL cells, which displayed the highest levels of pAKT among the three cell lines.

**DISCUSSION**

Through combined PTEN silencing and mutational activation of AKT1 in the context of the BRAFV600E oncprotein kinase and INK4A-ARF silencing, we have established an autochthonous model of spontaneous lung and brain metastases that is similar to the human disease. Furthermore, we provide compelling evidence that AKT1 activation plays a critical role in promoting melanoma metastasis to distant organs in vivo.
strated that the growth of BRAFV600E/PIK3CAH1047R melanomas depends on AKT signaling, but this dependency was not observed for the growth of BRAFV600E/PTENNull melanomas. These data suggest that there are likely to be specific isoforms of AKT differentially contribute to tumor growth and/or metastatic potential in melanoma and that activation of AKT2 or AKT3 following PTEN loss may oppose the activity of AKT1 resulting in the different phenotypes we observed. Activating mutations in AKT1 and AKT3 have been detected in human melanoma and are associated with disease progression (Davies et al., 2008; Shi et al., 2014). Future studies will compare the effects of AKT2 and AKT3 in this context.

In this study, we observed significant differences in mammalian target of rapamycin complex (mTORC) pathway components between our PTEN deficient and myrAKT1 expressing melanomas suggesting an elevation of this signaling downstream of AKT activation. Hyperactive mTORC signaling is disproportionately observed in melanomas (73%; 78/107) compared to benign nevi (4%; 3/67) (Karbowniczek et al., 2008). Deletion of Lkb1, a known negative regulator of mTORC signaling, in Kras mutant mouse melanocytes results in the formation of highly metastatic melanomas in vivo (Liu et al., 2012). More recently, loss of Lkb1 in the context of non-metastatic BRAFV600E/INK4A-ArfNull melanomas resulted in highly metastatic disease (Damsky et al., 2015). The pharmacological targeting of mTOR effectively blocks melanoma cell growth in vitro and in animal models (Dankort et al., 2009; Guba et al., 2002; Hidalgo and Rowinsky, 2000). Unfortunately, mTOR inhibitors have failed to demonstrate clinical efficacy against melanoma as mono-therapy and are currently being evaluated in combination with other therapies for this disease (Margolin et al., 2005). In line with increased mTORC signaling in melanoma metastasis, we found that total protein and phospho-protein levels of Rictor, an exclusive mTORC2 component, were significantly higher in myrAKT1-expressing melanomas than in PTEN-deficient melanomas (Figure 5; Figure S4). Combined inhibition of PI3K and mTOR has been shown to inhibit melanoma cell growth both in vitro and in vivo and interfere with angiogenesis (reviewed in Sznol et al., 2013). We observed that pharmacological inhibition of PI3K and mTOR also reduces melanoma cell migration. Taken together, our findings and those of others implicate increased mTORC signaling in promoting melanoma metastasis.

Figure 5. RPPA Analysis of Protein from Pten Null and myrAKT1 Melanomas

Protein was isolated from Cdkn2a\(^{+/−}\)/BRAF\(^{V600E}\)/Pten\(^{−/−}\) (Pten\(^{−/−}\)) and Cdkn2a\(^{+/−}\)/BRAF\(^{V600E}\)/myrAKT1 (myrAKT1) tumors; expression was assessed by RPPA. Log2 expression data were subjected to unsupervised clustering, and the results are presented as a heatmap. Tumors were labeled Pten\(^{−/−}\) (gray squares) or myrAKT1 (blue squares). The heatmap shown represents the proteins and phosphorylated proteins with a significant difference (p < 0.05 using unpaired t tests) between myrAKT1 and Pten\(^{−/−}\) tumors. An asterisk (*) denotes a primary tumor from a mouse with brain metastasis.

via a mechanism that is independent of, yet augmented by, PTEN silencing.

We observed dramatically increased distant metastasis in mice bearing tumors driven by activated AKT1 compared with Pten loss. Moreover, AKT1 activation cooperated with Pten loss to accelerate tumor formation and further increase dissemination to the lungs and brain. Our data suggest that aberrant AKT1 signaling in melanoma extends beyond the modulation of PIP3 levels by PTEN and PI3K. Our previous work demonstrated that the growth of BRAF\(^{V600E}\)/PIK3CA\(^{H1047R}\) melanomas depends on AKT signaling, but this dependency was not observed for the growth of BRAF\(^{V600E}\)/PTEN\(^{Null}\) melanomas (Marsh Durban et al., 2013). Our current study builds on these findings and demonstrates that BRAF\(^{V600E}\)/PTEN\(^{Null}\)/INK4A-ARF\(^{Null}\) melanomas are not significantly metastatic (-10%) compared to controls, while ectopic expression of myrAKT1 strongly potentiates metastasis in BRAF\(^{V600E}\)/INK4A-ARF\(^{Null}\) melanomas. These data suggest that there are likely to be PI3’-lipid signaling thresholds in melanoma cells that can confer different properties on the melanoma cell as demonstrated recently (Deuker et al., 2015).

Interestingly, PTEN deficient melanomas had significantly higher levels of phosphorylated AKT (Figures 3D, 4A, and 5) compared with tumors expressing myrAKT1 (Figures 3F, 3H, 4A, and 5) yet significantly fewer distant metastases. In mammals there are three isoforms of AKT, namely AKT1, AKT2 and AKT3. While the AKT isoforms share ~80% amino acid sequence identity, in vivo studies demonstrate that they have both redundant and non-overlapping functions (Gonzalez and McGraw, 2009). Compound AKT isoform knockout mice demonstrate that AKT1 exhibits functional redundancy with AKT2 and AKT3, but the reverse relationships lack full reciprocation (Dummler et al., 2006; Peng et al., 2003; Yang et al., 2005). Thus, for our studies of in vivo AKT signaling in melanoma, expression of activated AKT1 facilitates signaling in the broadest context. There have been conflicting reports regarding which isoforms of AKT are phosphorylated following PTEN loss in melanoma cells (Nogueira et al., 2010; Stahl et al., 2004). It is possible that signaling from AKT1 differs from the signaling that results from the activation of AKT isoforms with PTEN silencing. Some studies suggest that AKT isoforms have opposing functions with regards to tumorigenesis (Linnerth-Petrik et al., 2014; Maroulakou et al., 2007). It is possible that specific isoforms of AKT differentially contribute to tumor growth and/or metastatic potential in melanoma and that activation of AKT2 or AKT3 following PTEN loss may oppose the activity of AKT1 resulting in the different phenotypes we observed. Activating mutations in AKT1 and AKT3 have been detected in human melanoma and are associated with disease progression (Davies et al., 2008; Shi et al., 2014). Future studies will compare the effects of AKT2 and AKT3 in this context.
This model is ideally suited to further define the role of these proteins in melanoma metastasis, and future studies will focus on evaluating specific effectors downstream of AKT signaling in melanoma progression with the ultimate goal of identifying targets for therapies aimed at preventing or treating disseminated disease.

EXPERIMENTAL PROCEDURES

Vector Constructs

The retroviral vectors in this study are replication-competent avian leukemia virus, Bryan polymerase-containing vectors of envelope subgroup A (designated RCASBP(A) and abbreviated RCAS). RCAS-Cre and RCAS-myrAkt1 have been described previously (Aoki et al., 1998; VanBroeklin et al., 2010).

Cell Culture

DF-1 cells were grown in DMEM-high glucose media supplemented with 10% FBS (Invitrogen), 50 μg/ml gentamicin (Invitrogen) and maintained at 39 °C.

Migration Assays

Cell migration was evaluated for serum-starved cells with FBS as the chemo-attractant using a modified Boyden chamber assay (Cultrex 96 Well Cell Migration Assay, Trevigen) per the manufacturer’s specifications.

Virus Propagation

Virus infection was initiated by calcium phosphate transfection of plasmid DNA into DF-1 cells. Viral spread was monitored by GFP positive control transfection and expression of the p27 viral capsid protein by western blot.

Western Blotting

Tumor lysates were suspended in RPPA buffer (Tibes et al., 2006) with protease and phosphatase inhibitors (Pierce Biotechnology), separated on a 4%–20% Tris-glycine polyacrylamide gel, and transferred to nitrocellulose for immunoblotting. Detailed experimental procedures and antibodies used are described in Supplemental Experimental Procedures.

Viral Infections In Vivo

Infected DF-1 cells from a confluent culture in a 10-cm dish were trypsinized, pelleted, resuspended in 50 μl PBS, and placed on ice. Newborn mice were injected subcutaneously behind the ear with 50 μl suspended myrAkt1 cells and 50 μl suspended Cre cells.

Histology and Histochemical Staining

Mice were euthanized at their experimental endpoints set according to the guidelines of the University of Utah Institutional Animal Care and Use Committee. Mouse tissues were fixed in formalin overnight, dehydrated in 70% ethyl alcohol, and paraffin embedded. Sections were stained with H&E or left unstained for immunohistochemistry.

Immunohistochemistry

Tissue sections were hydrated, deparaffinized, and processed using the Biocare Medical HRP-Polymer system (Biocare Medical) according to the manufacturer’s recommendations. Detailed procedures and antibodies used are described in Supplemental Experimental Procedures.

Reverse-Phase Protein Array

Frozen tumor tissue was embedded in optimum cutting temperature (OCT) compound. H&E-stained slides were reviewed by an experienced dermatopathologist (Alexander J. Lazar) to identify areas that contained >70% tumor cells. Regions with extensive necrosis, fibrosis, or hemorrhage within the tumor specimens were excluded. The H&E slides were used as a guide to macrodissect the OCT blocks and isolate tumor-enriched regions for further analysis. 10- to 20-μm tumor shears were generated by cryostat and were used for protein and total RNA extraction. Proteins were isolated from the tumor shears, and RPPA was performed as previously described (Davies et al., 2009; Tibes et al., 2006).

Mice and Genotyping

Dct::TVA;Cdkn2alox/lox;Braflox/lox mice (designated as PtenWT) and Dct::TVA;Cdkn2alox/lox;Braflox/lox;Ptenlox/lox mice (referred to as Ptenlox/lox) were generated via crossing previously existing genetically engineered mice. All mice were maintained on a mixed C57Bl/6 and FVB/N background by random inter-breeding. DNA from tail biopsies was used to genotype for the Dct::TVA transgene, Cdkn2alox, Braflox and wild-type alleles as described previously (Dankort et al., 2007; VanBroeklin et al., 2010; Zheng et al., 2008).

Statistical Analysis

Censored survival data were analyzed using a log-rank test of the Kaplan-Meier estimate of survival. Densitometry of the western blot was performed using ImageJ (Schneider et al., 2012), and the data are presented as mean ± SEM. Student’s t test was used to compare migration between control and treated cells as well as protein expression levels between groups in the RPPA analysis. Protein-protein coefficients were determined by the Pearson correlation method, and the significance of the interactions was determined by the t statistic using R software. Unsupervised hierarchical clustering of mean-centered protein expression values was done using Cluster 2.1 and Treeview software.

Study Approval

All animal experimentation was performed in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved facilities at the University of Utah. All animal protocols were reviewed and approved prior to experimentation by the Institutional Animal Care and Use Committee at the University of Utah.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.09.057.

AUTHOR CONTRIBUTIONS

J.H.C. and J.P.R. contributed to the experimental design and performed the majority of the experiments. R.A.A., W.J.B., and D.A.K. performed immunohistochemistry and immunoblotting. G.C. and M.A.D. were responsible for the reverse-phase protein array experiments. A.H.G. provided pathological analysis. M.W.V., M.M., and S.L.H. were responsible for mouse model establishment, tumor induction, and characterization. J.H.C., M.M., and S.L.H. prepared the manuscript.

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Supplemental Information:

Detailed procedures for Western blot:

Tumor samples were flash frozen in liquid nitrogen and pulverized using a mortar and pestle. The pulverized tumor samples were resuspended in RPPA buffer with protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL, USA). Tumor samples in RPPA buffer were diluted in SDS-sample buffer and boiled for 10 minutes. The proteins were separated on a 4-20% Tris-glycine polyacrylamide gel, transferred to nitrocellulose, and incubated for 1 hour at room temperature in blocking solution (0.1% Tween-20 in 1X TBS with 5% nonfat dry milk, or 0.1% Tween-20 in 1X TBS with 5% BSA). Blots were immunostained with primary antibody at required dilution. All antibodies were diluted in TBS-T solution. Western blots were incubated in the primary antibody for 1 hour at room temperature with constant shaking and then washed in TBS-T wash buffer (0.1% Tween-20 in 1X TBS). The blots were then incubated with an anti-mouse IgG-horseradish peroxidase secondary antibody diluted 1:1000 (Sigma-Aldrich Corp., St. Louis, MO, USA) for 1 hour at room temperature. The blots were washed in TBS-T wash buffer, incubated with enhanced chemiluminescence solutions according to the manufacturer's specifications (Amersham, Piscataway, NJ USA), and exposed to film.

Primary antibodies used for Western blot:

MyrAKT1 expression was detected using an antibody to the HA epitope (HA.11, Covance, Princeton, NJ USA), diluted 1:1000. Total AKT expression was detected using a pan AKT antibody (c67E7, Cell Signaling, Danvers, MA USA), diluted 1:1000. Total ERK expression was detected using a pan ERK antibody (137F5, Cell Signaling), diluted 1:1000. Phospho-AKT expression was detected using antibody p-AKT s473 (s473 D9E, Cell Signaling), diluted 1:1000. Phospho-ERK expression was detected using p-P44/42 T202/Y204 antibody (9101, Cell Signaling), diluted
1:1000. BRAF V600E expression detected using specific mutant antibody (VE1, Ventana, Tucson, AZ USA) diluted 1:1000. GAPDH used for loading control (MAB374, EMD Millipore, Billerica, MA USA), diluted 1:10,000.

**Detailed procedures for IHC:**

Tissue sections were hydrated and deparaffinized and antigen retrieval was performed in 1X “Rodent Decloaker” buffer (Biocare Medical, Concord, CA USA) by boiling for 20 minutes in a Decloaking Chamber (Biocare Medical). Sections were treated with 3% hydrogen peroxide and blocked in “Rodent Block” (Biocare Medical) for 30 minutes. Primary antibodies were diluted in Renaissance background reducing diluent (Biocare Medical). Sections were incubated overnight at 4°C and probed with “Mouse on Mouse HRP” polymer reagent (Biocare Medical). Visualization was carried out with DAB (Biocare Medical). Sections were counterstained with hematoxylin.

**Primary antibodies used for IHC:**

MyrAKT1 expression was detected using an antibody to the HA epitope (HA.11, Covance, Princeton, NJ USA), diluted 1:1000. Phospho-AKT expression was detected using antibody p-AKT S473 (S473 D9E, Cell Signaling), diluted 1:1000.

**RT-PCR and PCR:**

Total RNA and genomic DNA was extracted from mouse primary tumors using TRIzol reagent (Invitrogen) and chloroform. Precipitated nucleic acid was column purified using RNEasy Mini Kit (Qiagen). Samples were not subjected to DNAse treatment in order to preserve genomic DNA. SuperScript III First-Strand Synthesis System (Invitrogen) was employed to synthesize cDNA from total RNA. PCR reactions were set up using EconoTaq Plus Green 2x Master Mix (Lucigen). Primers used and expected amplicon sizes were as follows:
Cre Fwd Primer: GTTTCCCGCAGAACCTGAAGATGTTC
Cre Rev Primer: GTATCTCTGACCAGAGTCATCCTTAGCG
Expected amplicon: 661 bp

Pten exon 5 Fwd Primer: CGAACTGAGCTACATCCCGAGTTCATAC
Pten exon 5 Rev Primer: CTGAAGTACTATAGGTGCTGCTGCTGGAG
Expected amplicon for *Pten* wild-type: 1546 bp

Supplemental Figure Legends

**Figure S1.** *Pten* loss in melanomas from *Dct::TVA;Braf<sup>CA/CA</sup>;*Cdkn2a<sup>lox/lox</sup>*;*Pten<sup>lox/lox</sup> mice injected with *Cre* containing virus or *Cre* and *myrAkt1* containing viruses, Related to Figure 1A, B. A, C: Viral delivery and expression of Cre recombinase was confirmed by RT-PCR on mouse primary tumors induced by *Cre* injection or *Cre* and *myrAkt1* injection, respectively. B, D: Deletion of *Pten* exon 5 was determined by PCR amplifying genomic DNA from mouse primary tumors induced by *Cre* injection or *Cre* and *myrAkt1* injection, respectively.

**Figure S2.** Expression of *myrAKT1* in melanomas from *Dct::TVA;Braf<sup>CA/CA</sup>;*Cdkn2a<sup>lox/lox</sup>* mice injected with *myrAkt1* and *Cre* containing viruses, Related to Figure 1A. Virally delivered *myrAkt1* expression was detected by IHC using an antibody to the HA epitope tag on *myrAKT1*. Tumors are shown by mouse ID number. Scale bar represents 200 µm.

**Figure S3.** Expression of *myrAKT1* in melanomas from *Dct::TVA;Braf<sup>CA/CA</sup>;*Cdkn2a<sup>lox/lox</sup>*; *Pten<sup>lox/lox</sup> mice injected with *myrAkt1* and *Cre* containing viruses, Related to Figure 1B. Virally delivered *myrAkt1* expression was detected by IHC using an antibody to the HA epitope tag on *myrAKT1*. Tumors are shown by mouse ID number. Scale bar represents 200 µm.

**Figure S4.** Protein and phospho-protein levels from RPPA, Related to Figure 5. Scatter plots show raw expression data from *Pten<sup>-/-</sup>* and *myrAKT1* tumors according to targets that were significantly different (p < 0.05 using unpaired t tests) between the two tumor groups. Solid line within data points equals geometric mean. Error bars represent SEM. Significant *P* values are denoted as follows: * for < 0.05, ** for < 0.01, and *** for < 0.001.
Cre Fwd Primer: GTTTCCCGCAGAACCTGAAGATGTTC
Cre Rev Primer: GTATCTCTGACCAGAGTCTCCTTAGCG
Expected amplicon: 661 bp

Pten exon 5 Fwd Primer: CGAACTGAGCTACATCCAGAGTTGCAT
Pten exon 5 Rev Primer: CTGGAAGTACTATAGGTGCCTGCTGGAG
Expected amplicon for Pten wild-type: 1546 bp

**Supplemental Figure Legends**

**Figure S1.** Pten loss in melanomas from Dct::TVA;Braf\(^{CA/CA}\);Cdkn2a\(^{lox/lox}\);Pten\(^{lox/lox}\) mice injected with Cre containing virus or Cre and myrAkt1 containing viruses, Related to Figure 1A, B. A, C: Viral delivery and expression of Cre recombinase was confirmed by RT-PCR on mouse primary tumors induced by Cre injection or Cre and myrAkt1 injection, respectively. B, D: Deletion of Pten exon 5 was determined by PCR amplifying genomic DNA from mouse primary tumors induced by Cre injection or Cre and myrAkt1 injection, respectively.

**Figure S2.** Expression of myrAKT1 in melanomas from Dct::TVA;Braf\(^{CA/CA}\);Cdkn2a\(^{lox/lox}\) mice injected with myrAkt1 and Cre containing viruses, Related to Figure 1A. Virally delivered myrAkt1 expression was detected by IHC using an antibody to the HA epitope tag on myrAKT1. Tumors are shown by mouse ID number. Scale bar represents 200 µm.

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Supplemental Figure Legends

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Expected amplicon for Pten wild-type: 1546 bp

Supplemental Figure Legends

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Supplemental Figure Legends

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**Figure S2.** Expression of myrAKT1 in melanomas from *Dct::TVA;Braf<sup>CA/CA</sup>;Cdkn2a<sub>lox/lox</sub> mice injected with myr*Akt1 and *Cre* containing viruses, Related to Figure 1A. Virally delivered myr*Akt1 expression was detected by IHC using an antibody to the HA epitope tag on myrAKT1. Tumors are shown by mouse ID number. Scale bar represents 200 µm.

**Figure S3.** Expression of myrAKT1 in melanomas from *Dct::TVA;Braf<sup>CA/CA</sup>;Cdkn2a<sub>lox/lox</sub>;*Pten<sub>lox/lox</sub> mice injected with myr*Akt1 and *Cre* containing viruses, Related to Figure 1B. Virally delivered myr*Akt1 expression was detected by IHC using an antibody to the HA epitope tag on myrAKT1. Tumors are shown by mouse ID number. Scale bar represents 200 µm.

**Figure S4.** Protein and phospho-protein levels from RPPA, Related to Figure 5. Scatter plots show raw expression data from *Pten<sup>-/-</sup>* and myrAKT1 tumors according to targets that were significantly different (p < 0.05 using unpaired t tests) between the two tumor groups. Solid line within data points equals geometric mean. Error bars represent SEM. Significant *P* values are denoted as follows: * for < 0.05, ** for < 0.01, and *** for < 0.001.
### A

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Treatment</th>
<th>Image</th>
<th>Count ± SD</th>
<th>p-value</th>
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<tbody>
<tr>
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<td>7.0 ± 1.0</td>
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<td>M14</td>
<td>DMSO</td>
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<td>A375</td>
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<td></td>
<td>NVP-BEZ-235</td>
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### B

<table>
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<th>AKT</th>
<th>GAPDH</th>
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<td>CAACL</td>
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<td><img src="image14.png" alt="Image" /></td>
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<tr>
<td>M14</td>
<td>+</td>
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<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
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</table>
**Figure S5. Dual PI3K/mTOR inhibition reduces melanoma cell migration, Related to Figures 4A-B, 5.**

**A:** Trevigen’s Cultrex® 96 Well Cell Migration Assay was used per the manufacturer’s specifications with FBS as the chemoattractant. The cells were treated with DMSO or 1 µM NVP-BEZ-235 as indicated. Migration was assessed after 48 hours. Representative images are shown. Quantification of the number of migrated cells is indicated below each image. Data represents the mean ± SEM. P-values are indicated for each cell line comparison.

**B:** Pharmacological inhibition of PI3K and mTOR reduces AKT phosphorylation. Lysates of A375, CACL, and M14 melanoma cells, treated with either DMSO or 2 µmol/L BEZ-235 for 24 hours as indicated, were analyzed by immunoblotting with the indicated antisera.
### Supplementary Table 1. Summary of tumor formation, Related to Figure 1A-B.

The mean survival (days) and tumor incidence (fraction of tumor-bearing mice per cohort) are listed for each cohort according to genetic background and virally delivered genes.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene(s) delivered</th>
<th>Tumor Incidence</th>
<th>Mean survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Braf&lt;sup&gt;CA&lt;/sup&gt;;Cdkn2a&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
<td>Cre</td>
<td>0/40</td>
<td>N/A</td>
</tr>
<tr>
<td>Dct::TVA;Braf&lt;sup&gt;CA&lt;/sup&gt;;Cdkn2a&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
<td>Cre</td>
<td>16/34</td>
<td>88.9 ± 8.6</td>
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<tr>
<td>Braf&lt;sup&gt;CA&lt;/sup&gt;;Cdkn2a&lt;sup&gt;lox/lox&lt;/sup&gt;;Pten&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
<td>Cre</td>
<td>0/40</td>
<td>N/A</td>
</tr>
<tr>
<td>Dct::TVA;Braf&lt;sup&gt;CA&lt;/sup&gt;;Cdkn2a&lt;sup&gt;lox/lox&lt;/sup&gt;;Pten&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
<td>Cre</td>
<td>24/24</td>
<td>57.8 ± 3.4</td>
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<tr>
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<td>N/A</td>
</tr>
<tr>
<td>Dct::TVA;Braf&lt;sup&gt;CA&lt;/sup&gt;;Cdkn2a&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
<td>Cre + myrAkt1-&lt;i&gt;HA&lt;/i&gt; myrAKT1-HA positive*</td>
<td>24/27/18</td>
<td>65.3 ± 4.7/58.9 ± 3.5</td>
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<tr>
<td>Dct::TVA;Braf&lt;sup&gt;CA&lt;/sup&gt;;Cdkn2a&lt;sup&gt;lox/lox&lt;/sup&gt;;Pten&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
<td>Cre + myrAkt1</td>
<td>14/14</td>
<td>42.1 ± 2.8</td>
</tr>
</tbody>
</table>

*HA positive by IHC.

### Supplementary Table 2. Protein expression from RPPA for PTEN<sup>Null</sup> and myrAKT1-expressing melanomas, Related to Figure 5.

Expression of 131 protein and phosphoprotein epitopes assessed by RPPA listed for PTEN<sup>Null</sup> and myrAKT1-expressing melanomas with associated p-values for differences in expression between the two groups.
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<th>Mean survival (days)</th>
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</thead>
<tbody>
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<td>Cre</td>
<td>0/40</td>
<td>N/A</td>
</tr>
<tr>
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<td>Cre</td>
<td>16/34</td>
<td>88.9 ± 8.6</td>
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<tr>
<td>Braf\textsuperscript{CA}, Cdkn2a\textsubscript{lox/lox}, Pten\textsubscript{lox/lox}</td>
<td>Cre</td>
<td>0/40</td>
<td>N/A</td>
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<tr>
<td>Dct::TVA; Braf\textsuperscript{CA}, Cdkn2a\textsubscript{lox/lox}, Pten\textsubscript{lox/lox}</td>
<td>Cre</td>
<td>24/24</td>
<td>57.8 ± 3.4</td>
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<tr>
<td>Dct::TVA; Braf\textsuperscript{CA}, Cdkn2a\textsubscript{lox/lox}</td>
<td>myrAkt1</td>
<td>0/21</td>
<td>N/A</td>
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<tr>
<td>Dct::TVA; Braf\textsuperscript{CA}, Cdkn2a\textsubscript{lox/lox}</td>
<td>Cre + myrAkt1-HA, myrAKT1-HA positive*</td>
<td>24/27</td>
<td>65.3 ± 4.7</td>
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<tr>
<td>Dct::TVA; Braf\textsuperscript{CA}, Cdkn2a\textsubscript{lox/lox}, Pten\textsubscript{lox/lox}</td>
<td>Cre + myrAkt1</td>
<td>14/14</td>
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