Methods

Mice and Genotyping

Dct::TVA;Braf^{CA}*;Cdkn2a*$^{lox/lox}$*;Pten*$^{lox/lox}$ mice were maintained on a mixed C57Bl/6 and FVB/N background by random interbreeding. DNA from tail biopsies was used to genotype for the *TVA* transgene, *Braf*^{CA}, *Cdkn2a*$^{lox/lox}$,*Pten*$^{lox/lox}$, and wild-type alleles as described (51-53). Both male and female newborn through adult mice were used in this study. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah.

Viral Constructs and Propagation

The Gateway compatible RCAS destination vector has been described (54). RCAS-Cre (52) and RCAS-myrAKT1 also have been described (17,19). RCAS-myrAKT1 was used as a template to generate the *Akt1* mutant constructs. Mouse melanocyte cDNA and *Akt2*-specific primers were used to amplify *Akt2* via PCR. The product was TOPO cloned into a gateway compatible pCR8 TOPO vector, then subcloned into the RCAS destination vector. RCAS-AKT2 was used as a template to generate the *Akt2* mutant constructs. Mouse myr*Akt3* was amplified via PCR reaction from *Akt3* cDNA (Dharmacon, Lafeyette, CO) and *Akt3*-specific primers. The product was TOPO cloned into a gateway compatible pCR8 TOPO vector, then subcloned into the RCAS destination vector. RCAS-myrAKT3 was used as a template to generate the *Akt3* mutant constructs. All mutants were engineered with an N-terminal HA-epitope tag. For E17K mutants, the E17K substitution was engineered into the forward primer of each paralog. PCR overlap extension was performed to engineer E40K, Q79K, and K179M mutants, which were subcloned into

gateway compatible pCR8 TOPO vectors (Thermo Fisher, Waltham, MA), then gateway cloned into the RCAS destination vector. The K179M mutant was generated using RCAS-AKT1^{E17K} as a template. The PCR product was subcloned into a pCR8 TOPO vector (Thermo Fisher), then gateway cloned into the RCAS destination vector. All viral vectors were verified via Sanger Sequencing. Primer sequences are available upon request. Viral production was initiated by calcium phosphate transfection into DF-1 cells. Viral spread was monitored by expression of the p27 viral capsid protein as detected by immunoblot.

Cell Culture

DF-1 cells were grown in DMEM-high glucose media (Thermo Fisher) supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA) and 0.5 µg/mL Gentamicin (Thermo Fisher), and maintained at 39°C. Yale University Mouse Melanoma cells (YUMM 1.1) were grown in F12/DMEM media (Thermo Fisher) supplemented with 10% FBS, 1 µg/mL penicillin-streptomycin (Thermo Fisher) and 1 µg/mL non-essential amino acids (Thermo Fisher), and maintained at 37°C. Cell line 5610 was established by isolating *Dct::TVA;*BRAF^{V600E}*;Cdkn2a*$^{-/-}$ primary tumor tissue from a mouse during necropsy. The tissue was minced and incubated in F12/DMEM media (Thermo Fisher) supplemented with 10% FBS, 1 µg/mL penicillin-streptomycin and 1 µg/mL non-essential amino acids, and maintained at 37°C. Cell line 9678 was established from a *Dct::TVA;*BRAF^{V600E} *;Cdkn2a*$^{-/-}$*;Pten*$^{-/-}$ primary tumor, and cell line 7788 was established from a *Dct::TVA;*BRAF^{V600E} *;Cdkn2a*$^{-/-}$*;Pten*$^{-/-}$*;*AKT1^{E17K} primary tumor using the same tissue isolation procedure and cell culture conditions as described for 5610.

Viral Infections (*In Vivo*)

Infected DF-1 cells from a confluent culture in a 10 cm dish were trypsinized, pelleted, resuspended in 100 µL of Hank's Balanced Salt Solution (HBSS) (Thermo Fisher), and placed on ice. Newborn mice were injected subcutaneously behind each ear with 50 µL of suspended RCAS-AKT cells with or without RCAS-Cre cells.

Viral Infections (*In Vitro*)

YUMM 1.1 cells were transfected with pcDNA3.1-*TVA* (55) containing the hygromycin B-resistance gene to generate YUMM1.1-TVA cells. TVA-positive clones (YUMM 1.1 TVA+) were selected in 300 µg/mL Hygromycin B (Thermo Fisher). Supernatant from DF-1 cells producing RCAS-AKT1 or RCAS-AKT1^{E17K} was used to infect YUMM 1.1 TVA+ cells. Expression of AKT1 and AKT1^{E17K} in YUMM 1.1 cells was confirmed by immunoblot and/or immunofluorescence. To generate the 5610^{E17K} cell line, AKT1^{E17K} was gateway cloned from pCR8 TOPO to a pHIV EF1a-Luciferase-IRES-ZsGreen destination vector (kind gift from Bryan Welm) to generate pHIV CMV-HA-AKT1^{E17K}-EF1a-*Z*sGreen. The lentiviral vector pHIV CMV-HA-AKT1^{E17K}-EF1a-*Z*sGreen, along with packaging plasmids psPAX2 (#12260 Addgene, Cambridge, MA) and pCMV-VSV-G (#8454 Addgene) were transfected into 293FT cells using the calcium phosphate method. Supernatant from these cells containing virus was then used to infect the 5610 parental cell line. 5610^{E17K} mouse melanoma cells were sorted for ZsGreen using a Propel Labs Avalon at The Flow Cytometry Shared Resource Laboratory at the University of Utah. Expression of AKT1^{E17K} in 5610 cells was confirmed by immunoblot.

Reverse Phase Protein Array (RPPA)

Frozen primary tumor samples were embedded in optimal cutting temperature compound (OCT). A 5 µm H&E-stained slide was prepared from each sample and reviewed by a pathologist for regions containing 70% or more viable tumor. Marked H&E slides were then used to guide macrodissection of viable tumor from the OCT blocks. Extraction of protein from tumor tissue was performed as previously described (6). RPPA analysis was performed at the MD Anderson RPPA Core Facility. Lysates were arrayed on nitrocellulose-coated slides, probed with 304 antibodies by tyramide-based signal amplification, and visualized by DAB colorimetric reaction. Array-Pro Analyzer was used to quantify 16-bit TIFF images produced by slides scanned on a flatbed scanner. Relative protein levels were determined by interpolation of each dilution curve from the standard curve of the slide. All data points were normalized for protein loading and transformed to a linear value. Normalized linear values were transformed to Log2 values, then median-centered for hierarchical clustering analysis. The accession number for data reported is GEO: GSE123182.

RNA Sequencing

Frozen primary tumor samples embedded in OCT were macrodissected as per the methods described for RPPA. For RNA extraction and DNase treatment, the Roche High Pure miRNA Isolation Kit was used in accordance with the manufacturer's protocol (5080576001). RNA sequencing was performed at the High-Throughput Genomics and Bioinformatics Analysis Core at the University of Utah. RNA libraries were prepared using the Illumina TruSeq Stranded Total RNA Kit with Ribo-Zero Gold. Total RNA samples (100-500 ng) were hybridized with Ribo-Zero Gold to substantially deplete cytoplasmic and mitochondrial rRNA from the samples. Stranded RNA sequencing libraries were

prepared using the Illumina TruSeq Stranded Total RNA Kit with Ribo-Zero Gold per the manufacturer's instructions (RS-122-2301 and RS-122-2302). Purified libraries were qualified on an Agilent Technologies 2200 TapeStation using a D1000 ScreenTape assay (cat# 5067-5582 and 5067-5583). The molarity of adapter-modified molecules was defined by quantitative PCR using the Kapa Biosystems Kapa Library Quant Kit (cat#KK4824). Individual libraries were normalized to 10 nM and equal volumes were pooled in preparation for Illumina sequence analysis. DNA sequences were determined using the HiSeq 50 Cycle Single-Read Sequencing version 4. Sequencing libraries (25 pM) were chemically denatured and applied to an Illumina HiSeq v4 single read flow cell using an Illumina cBot. Hybridized molecules were clonally amplified and annealed to sequencing primers with reagents from an Illumina HiSeq SR Cluster Kit v4-cBot (GD-401-4001). Following transfer of the flowcell to an Illumina HiSeq 2500 instrument (HCSv2.2.38 and RTA v1.18.61), a 50 cycle single-read sequence run was performed using HiSeq SBS Kit v4 sequencing reagents (FC-401-4002). The accession number for data reported is GEO: GSE122781.

TCGA Data Analysis

Data were collected from TCGA-SKCM using the TCGAbiolinks R package via Bioconductor. These data were stratified into 66 primary melanomas from patients who, at the time of this publication, had not experienced a metastatic event. These were compared with 43 melanomas (4 primary tumor, 7 regional cutaneous or subcutaneous tumor, 27 regional lymph node, 5 distant metastasis) from patients who developed brain metastases.

Spheroid Formation and Invasion Assay

Ninety-six well plates were coated with 50 µL of 3 mg/mL Matrigel (Corning, Corning, NY) in serum-free F12/DMEM media (Thermo Fisher) supplemented with 1 µg/mL penicillin-streptomycin (Thermo Fisher) and 1 µg/mL non-essential amino acids (Thermo Fisher). The plates were incubated at 37°C for 2 hours. One thousand YUMM 1.1 control, AKT1, or AKT1^{E17K}-expressing cells in F12/DMEM media supplemented with 10% FBS (Atlanta Biologicals) and 3 mg/mL Matrigel (Corning), were added to the base layer of Matrigel. Plates were incubated at 37°C for 5 days. Imaging of spheroids was performed using an EvosFL microscope and ImageJ was used to quantify spheroid formation and invasion.

Inhibitors

The AKT inhibitor (GSK-2141795), FAK inhibitor (PF-573228), and Src inhibitor (Dasatinib) were purchased from Selleck Chemical (Houston, TX) and formulated in DMSO. Each drug was added to F12/DMEM media (Thermo Fisher) for a final concentration of 0.1% DMSO. F12/DMEM media was supplemented with 2% FBS (Atlanta Biologicals), 1 µg/mL penicillin-streptomycin (Thermo Fisher), and 1 µg/mL non-essential amino acids (Thermo Fisher). YUMM 1.1 AKT1^{E17K}-expressing cells were incubated in 500 nM or 1000 nM of GSK-2141795, 100 nM or 500 nM of PF-573228, 10 nM or 50 nM of Dasatinib, or 0.1% DMSO vehicle control. Media was refreshed daily. Cells were lysed on day 5 with 100 mM Tris-HCL, 4% SDS, 20% glycerol, and 10% DTT. Lysates were separated by SDS-PAGE and assessed by immunoblot.

Transwell Invasion Assay

YUMM 1.1 parental, AKT1, and AKT1^{E17K} -expressing cells were plated in F12/DMEM serum-free media (Thermo Fisher) supplemented with 1% BSA (Cell Signaling

Technology, Danvers, MA), 1 μg/mL penicillin-streptomycin (Thermo Fisher) and 1 μg/mL non-essential amino acids (Thermo Fisher), and maintained at 37°C for 24 hours. Transwells (6.5 mm, 8 μm pores) (Corning) were prepared by adding 50 μL of 2 mg/mL Matrigel (Corning) suspended in F12/DMEM serum-free media (Thermo Fisher) supplemented with 1% BSA (Cell Signaling Technology), 1 μg/mL penicillin-streptomycin (Thermo Fisher) and 1 μg/mL non-essential amino acids (Thermo Fisher), and maintained at 37°C for 2 hours. Fifty-thousand serum-starved YUMM 1.1 control, AKT1, or $AKT1^{E17K}$-expressing cells were then re-suspended F12/DMEM serum-free media (Thermo Fisher) supplemented with 1% BSA (Cell Signaling Technology), 1 μg/mL penicillin-streptomycin (Thermo Fisher), 1 μg/mL non-essential amino acids (Thermo Fisher), and 0.1% DMSO (vehicle control). A subset of YUMM $AKT1^{E17K}$-expressing cells were resuspended in F12/DMEM media with 1000 nM of GSK-2141795, 500 nM of PF-573228, or 50 nM of Dasatinib. All cells were plated on the surface of the Matrigel in transwells and 800 μL of F12/DMEM media supplemented with 10% FBS (Atlanta Biologicals), 1 μg/mL penicillin-streptomycin (Thermo Fisher), and 1 μg/mL non-essential amino acids (Thermo Fisher) was added to the bottom chamber as a chemoattractant. Media on the apical surface of transwells was refreshed daily. On day 5, transwells were rinsed in PBS, incubated in 100% methanol for 10 minutes, and stained with crystal violet for 10 minutes. The Matrigel and cells on the apical surface of the transwells were removed using a cotton swab. Cells on the basolateral surface of each well were imaged, then incubated in 20% acetic acid in H_2O for 10 minutes on a rotating orbital to dissolve the crystal violet. Absorbance was read on a microplate reader at 590 nM.

Transwell Migration Assay

The experimental methods for YUMM 1.1 cells followed those of the Transwell Invasion Assay with the exception of the use of Matrigel, 0.1% DMSO-supplemented media, and the use of compounds to inhibit AKT, FAK, and Src. Identical quantitative analyses were performed on fixed and stained cells.

Tail Vein Injections

Four-to-six week old NOD *scid* gamma (NSG) mice (Jackson Laboratory, Bar Harbor, ME) were injected into the tail vein with 2.5 x 10^5 mouse melanoma cells suspended in 150 µL of PBS. At the experimental endpoint, brains and lungs were isolated and processed as described above.

Intracranial Injections

Newborn NSG mice were intracranially injected into the right cerebrum with 1.0 x 10^4 mouse melanoma cells suspended in 5 µL of Hank's Balanced Salt Solution (HBSS) (Thermo Fisher) using a gas-tight Hamilton syringe. At the experimental endpoint, brains were isolated and processed as described above.

Immunohistochemistry

Tissue sections from formalin-fixed paraffin embedded blocks were deparaffinized at 65˚C for 10 minutes, incubated in xylene, and rehydrated using decreasing concentrations of ethanol. Antigen retrieval was performed in a decloaking chamber at 120˚C for 20 minutes using citrate buffer (pH 6.0) for HA, P-ERK, S100, PTEN, P-AKT, and Ki67, or EDTA (pH 8.0) for Paxillin, P-Paxillin, FAK, and P-FAK. Peroxidase activity was quenched in 3% H_2O_2 for 10 minutes and slides were incubated in 5% normal goat serum in TBS-T (0.05% Tween-20) for 1 hour inside a humidity chamber. Anti-rabbit

primary antibodies were diluted in SignalStain Antibody Diluent (Cell Signaling Technology), added to slides, and incubated overnight at 4˚C. Slides were incubated in SignalStain Boost Detection Reagent (Cell Signaling Technology) for 30 minutes in a humidity chamber and the SignalStain DAB Substrate Kit (Cell Signaling Technology) was used to detect the presence of each epitope. Slides were counterstained in hematoxylin and dehydrated with increasing concentrations of alcohol and xylene prior to the addition of coverslips. Antibodies used: HA (1:500; MMS101P Biolegend, San Diego, CA), S100 (1:100; RB-9018 Thermo Fisher), Ki67 (1:300; UM800033 Origene, Rockville, MD), Paxillin (1:100; STJ94969 Antibodyplus, Brookline, MA), Phospho-Paxillin (Y31) (1:100; STJ90380 Antibodyplus), Phospho-ERK1/2 (T202/Y204) (1:400; 4370 Cell Signaling Technology), PTEN (1:125; 9188 Cell Signaling Technology), Phospho-AKT (S473) (1:100; 3787 Cell Signaling Technology), FAK (1:100; 3285 Cell Signaling Technology), Phospho-FAK (1:50; Y397) (3283 Cell Signaling Technology).

Immunoblotting

Cell lysates were suspended in 100 mM Tris-HCL, 4% SDS, 20% glycerol, and 10% DTT. To generate lysates from frozen primary tumors, tissues were pulverized in liquid nitrogen using a mortar and pestle and samples were resuspended in RPPA buffer (8) with protease and phosphatase inhibitors (Pierce Biotechnology). All lysates were incubated at 95˚C for 10 minutes, separated on an 8-16% Tris-glycine polyacrylamide gel (Thermo Fisher Scientific), and transferred to nitrocellulose for immunoblotting. Nitrocellulose membranes were incubated in blocking solution composed of 0.1% Tween-20 in 1X TBS with 5% nonfat dry milk for HA, GAPDH, and P-AKT (T308), or 5% BSA (Cell Signaling Technology) for all other antibodies. Blots were immunostained in the primary antibody

diluted 1:1,000 (or 1:10,000 for Gapdh) in TBS-T for 1 hour with constant shaking and washed in TBS-T. Blots were then incubated in anti-mouse IgG-HRP or anti-rabbit IgG-HRP secondary antibody diluted 1:1,000 in TBS-T for 1 hour with constant shaking and washed in TBS-T. Enhanced chemiluminescence (Amersham) was used according to the manufacturer's specifications and blots were exposed to film. Antibody list: p27-HRP (10100770; Charles River Laboratories), HA (MMS-101P; Biolegend), GAPDH (MAB374; MilliporeSigma), Paxillin (STJ94969; Antibodyplus), Phospho-Paxillin (Y31) (STJ90380; Antibodyplus), FAK (3285; Cell Signaling Technology), Phospho-FAK (Y397) (3283; Cell Signaling Technology), Phospho-FAK (Y925) (3284; Cell Signaling Technology), AKT (4691; Cell Signaling Technology), Phospho-AKT (T308) (13038; Cell Signaling Technology), Phospho-AKT (S473) (3787; Cell Signaling Technology), PRAS40 (2691; Cell Signaling Technology), Phospho-PRAS40 (Y246) (2997; Cell Signaling Technology), GSK3β (12456; Cell Signaling Technology), Phospho-GSK3β (S9) (5558; Cell Signaling Technology), anti-mouse-HRP (7076; Cell Signaling Technology), Anti-rabbit-HRP (7074; Cell Signaling Technology).

Immunofluorescence

Fifty-thousand YUMM 1.1 control or $AKT1^{E17K}$-expressing cells were plated into 35 mm glass bottom plates (MatTek, Ashland, MA) suspended in F12/DMEM media (Thermo Fisher) supplemented with 10% FBS (Atlanta Biologicals), 1 μg/mL penicillin-streptomycin (Thermo Fisher) and 1 μg/mL non-essential amino acids (Thermo Fisher), and maintained at 37°C for 24 hours. Plates were fixed in 3% paraformaldehyde in PBS for 20 minutes, washed in PBS, and incubated for 30 minutes in blocking buffer composed of 1% BSA (Cell Signaling Technology) and 0.15% glycine (Thermo Fisher) in PBS. Cells

were permeabolized with 0.1% triton (Sigma-Aldridge, St. Louis, MO) in PBS for 15 minutes, washed in PBS, and incubated for 30 additional minutes in blocking buffer. Plates were then incubated in 15% goat serum (Cell Signaling Technology) in PBS for 30 minutes, followed by the addition of primary anti-HA and anti-paxillin antibodies diluted in 5% goat serum for 1 hour and washed with PBS. Incubation with secondary fluorophore 488 and 568-conjugated antibodies diluted in 5% goat serum was performed for 1 hour followed by washes with PBS. DAPI stain (Thermo Fisher) at 5 ng/mL in PBS was added for 1 minute followed by washes with PBS. Plates were left in PBS solution and images were acquired using a Nikon Ti spinning disc confocal/TIRFM microscope. Antibodies used: HA (1:1,600; 3724 Cell Signaling Technology), Paxillin (1:1,000; 610051 BD Biosciences, Franklin Lakes, NJ), Anti-mouse Alexa Fluor 488 (1:1,000; A-11029 Thermo Fisher), Anti-rabbit Alexa Fluor 568 (1:1,000; A-11036 Thermo Fisher).

Statistical Methods

Mouse survival data was analyzed using a log-rank (Mantel-Cox) test of the Kaplan-Meier estimate of survival; a Fisher's exact test was used to determine differences in metastasis between cohorts. For RPPA analysis, heat-maps were generated in Excel and rows were scaled using z-scores ($\frac{x-\mu}{\sigma}$); an unpaired *t* test was used to calculate differences between groups. Densitometry measurements were performed using ImageJ and protein levels were normalized to GAPDH; the data are represented as mean ± S.E.M. For RNA sequencing analyses, the Bioconductor package DESeq2 in RStudio was used to generate PCA plots, volcano plots, and heat-maps with dendrogram clustering. The Limma package vignette was used to perform mean-variance remodeling (voom-transformation) and convert read counts into logCPM for visual comparison of individual

genes. A Benjamini-Hochberg correction was applied to correct for multiple testing and generate adjusted p-values for individual gene comparisons using a false discovery rate threshold of 0.05 in RStudio. For melanoma spheroid formation and invasion assays, unpaired *t* tests were performed using the average number of colonies formed among all wells for each condition and the average percentage of colonies to form invasive protrusions among all wells for each condition. Data are represented as mean ± S.E.M. A p value of < 0.05 was considered statistically significant for all analyses. The asterisks shown in figures correspond to P values as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).