Akt3 and Mutant ^{V600E}B-Raf Cooperate to Promote Early Melanoma Development

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Abstract

B-Raf **is the most mutated gene in melanoma; however, the mechanism through which it promotes early melanomas remains uncertain. Most nevi contain activated ^{V600E}B-Raf but few develop into melanoma, and expression in melanocytes is inhibitory with low protein levels present in surviving cells, suggesting unknown cooperative oncogenic events are necessary for melanoma development. Because many melanomas have ^{V600E}B-Raf and active Akt3, it is possible that these proteins cooperatively facilitate melanocyte transformation. In this study, Akt3 is shown to phosphorylate ^{V600E}B-Raf to lower its activity as well as that of the downstream mitogen-activated protein kinase (MAPK) pathway to levels promoting early melanoma development. Expression of active Akt3 in early melanoma cells containing ^{V600E}B-Raf reduced MAPK signaling and promoted anchorage-independent growth. Furthermore, expression of both ^{V600E}B-Raf and active Akt3 in melanocytes promoted a transformed phenotype. Mechanistically, aberrant Akt3 activity in early melanomas serves to phosphorylate Ser^{364} and Ser^{428} on ^{V600E}B-Raf to reduce activity of ^{V600E}B-Raf to levels that promote rather than inhibit proliferation, which aids melanocytic transformation. Inhibition of ^{V600E}B-Raf or Akt3 in advanced melanoma cells in which both pathways were active reduced anchorage-independent growth and tumor development in a cooperatively acting manner. Inhibition of Akt3 alone in these cells led to increased MAPK signaling. In summary, these results suggest that activating B-Raf mutations initially promote nevi development, but the resulting high, intense activation of the MAPK pathway inhibits further tumor progression requiring Akt3 activation to bypass this barrier and aid melanoma development.** [Cancer Res 2008;68(9):3429–39]

Introduction

Despite being the least prevalent type of skin cancer, malignant melanoma is by far the deadliest due to its highly metastatic nature (1). Phosphatidylinositol-3-OH (PI3) and mitogen-activated protein kinase (MAPK) pathways play prominent roles in melanoma development by regulating diverse cellular processes including cellular proliferation, survival, invasion, and angiogenesis (2–4). The mechanism through which these pathways interact or cooperate to promote melanoma development remains to be fully elucidated (2).

Increased activity of the PI3K pathway occurs in ~70% of sporadic melanomas due to loss of PTEN and/or increased expression of Akt3 resulting from elevated gene copy number (5). Highest level of Akt3 activity occur in metastatic melanomas, deregulating apoptotic signaling and promoting chemoresistance, making it an attractive therapeutic target for metastatic disease (6–8). Targeting Akt3 decreases tumorigenesis by increasing cellular apoptosis in advanced stage melanomas, but it is not known whether it plays a second independent role in early melanoma development (7, 9).

The MAPK pathway is activated through Ras mutations in 10% to 15% and *B-Raf* mutations in ~60% of melanomas (10–16). A single-base mutation converting T to A at nucleotide 1,799 of *B-Raf*, substituting a valine for a glutamic acid at codon 600 (V600E) in exon 15, is the most prominent mutation (12) and is acquired but not inherited during development of sporadic melanomas (17–19). Because *B-Raf* is the most mutated gene in melanomas, it is an attractive therapeutic target for melanoma patients (20).

Although ^{V600E}B-Raf regulates melanoma cellular proliferation (21–23), angiogenesis (21), survival (22, 23), invasion, and metastasis (24, 25), it alone is not sufficient for melanoma development. Ectopic expression of Ras or Raf in primary or cancer cell lines causes cell cycle arrest or senescence (26), likely due to duration or level of MAPK pathway activation (27), which induces cyclin-dependent kinase (cdk) inhibitors (28, 29). Thus, other factors are necessary to facilitate melanoma tumor progression, likely involving loss of tumor suppressor genes or acquisition of cooperating oncogene activation (30–33). Possibly, deregulation of p53 (31, 33) and/or Rb pathway through p16^{INK4A} loss (30, 31) might be necessary. Another unexplored possibility might involve cooperation between ^{V600E}B-Raf and Akt3 signaling cascades, which are frequently activated in the same cells (3, 4). In support of this possibility, Akt has been shown to phosphorylate B-Raf to decrease its activity in normal cells; however, it is unknown whether this process could play any role in melanoma development (34–36).

In this study, ^{V600E}B-Raf and Akt3 are shown to cooperatively promote melanoma development. Akt3 is shown to phosphorylate mutant ^{V600E}B-Raf to reduce its and MAPK pathway activity to levels promoting rather than retarding melanocytic cell growth and transformation. Thus, these results suggest that activating ^{V600E}B-Raf mutation initially promotes nevi development, but the resulting high, intense activation of the MAPK pathway inhibits further tumor progression requiring Akt3 activation to phosphorylate ^{V600E}B-Raf to bypass this barrier and promote melanoma development.

Materials and Methods

Cell Lines and Culture Conditions

Normal human epidermal melanocytes (NHEM) and melanoma cell lines, WM35, UACC 903, and SK-MEL-24 cells were cultured as previously described (7, 37–39). Mouse melan-a melanocytes were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 200 nmol/L 12-*O*-tetradecanoylphorbol-13-acetate.

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In vitro Pharmacologic Agent Studies

The PI3K inhibitor LY-294002 (Qbiogene) was used to pharmacologically inhibit Akt activity. Briefly, 1×10^6 NHEM, WM35, SK-MEL-24, or 1.5×10^5 UACC 903 cells were grown in complete medium supplemented with FBS or growth factors. Two days later, cells were fed with serum/growth factor–free medium. Twenty-four hours later, cells were treated with 50 mmol/L LY-294002 or DMSO for 20 min followed by stimulation using serum or growth factors up to 180 min. At each time point, protein lysates were collected for Western blot analysis.

Western Blot Analysis

Protein lysates were collected, quantitated, and analyzed by Western blotting as detailed previously (21). Antibodies to pErk and pAkt Ser^{473} and caspase-3 from Cell Signaling Technologies; cyclin D1, p27 Kip1, α-enolase, anti-Hemagglutinin, and B-Raf from Santa Cruz Biotechnology; and Akt3 from Upstate Biotechnology were used.

Plasmid Constructs

Hemagglutinin-tagged wild-type and kinase-dead Akt3 (40) were subcloned (Eco RI and Xba I) into pcDNA3.1puro. A myristoylation tag was added by PCR to the NH_2 terminal of Akt3 and subcloned into the Eco RI and Xba I. Site-directed mutagenesis (Quick Change) was used to create E40K Akt3 mutant with primers TGGCTCATTCATAGGATATAAAAA-GAAACCTCAAGATGTGGATTT and AAATCCACATCTTGAGGTTTCTTTT-TATATCCTATGAATGAGCCA. For B-Raf constructs, Hemagglutinin-tagged wild-type B-Raf (provided by Dr. Ann Vojtek, University of Michigan, Ann Arbor, MI) was excised (Hind III/Eco RV) and subcloned into pcDNA6/V5-HisA (Invitrogen; ref. 41). Site-directed mutagenesis with primers GATTTTGGTCTAGCTACAGAGAAATCTCGATGGAGTGGG and CCCACTC-CATCGAGATTTCTCTGTAGCTAGACCAAAATC generated ^{V600E}B-Raf. Similarly, primers CGAGACCGATCCTCAGCAGCTCCCAATGTGCAT and ATGCACATTGGGAGCTGCTGAGGATCGGTCTCG generated S364A ^{V600E}B-Raf, and primers GCGAGAAAGGAAGTCAGCTTCATCCTCAGAA-GAC and GTCTTCTGAGGATGAAGCTGACTTCCTTTCTCGC generated S428A ^{V600E}B-Raf. Construct inserts were sequence verified. Plasmids were introduced into cell lines with the Amaxa Nucleofector. Reagent R, K17 program was used for UACC 903 cells, whereas NHEM reagent, U20 program was used for human melanocytes, WM35, and melan-a cells. Transfection efficiencies were determined to be ~60% using pMaxGFP as a green fluorescent protein expression plasmid control.

Anchorage-Independent Growth Studies

Plates were coated with PolyHEMA (12.0 mg/mL; Sigma) in 95% ethanol to assess anchorage-independent growth.

siRNA-mediated inhibition. Duplexed "Stealth" siRNA from Invitrogen was used for inhibiting protein expression as described previously (7, 9, 25, 39). siRNA (100 or 200 pmol) was nucleofected into 2×10^6 human melanocytes, UACC 903, or WM35 cells allowed to recover for 1.5 to 2 d. Cells were then trypsinized, and 2×10^4 cells were seeded into PolyHEMA-coated 96-well plates. Protein lysates for Western blot analysis was harvested. For UACC 903, 1×10^4 cells per well were seeded into DMEM supplemented with 10% FBS or 2×10^4 cells per well in serum-free conditions. Cell proliferation was quantified 3 d later by MTS assay (Promega).

Ectopic plasmid expression studies. Hemagglutinin-tagged Akt3 constructs (1.45 pmol pcDNA3.1puro Hemagglutinin wt-Akt3, E40K-Akt3, myr-Akt3, and dead-Akt3) were nucleofected alone or in combination with siRNA to PRAS40 (100 pmol) or a scrambled siRNA into 2×10^6 cells, 5×10^4 cells/50 μL plated into 96-well PolyHEMA-coated plates. Twenty-four hours later, cells were selected using 1.6 μg/mL puromycin-containing medium. After 7 d of selection, anchorage-independent growth was assessed by MTS assay.

Melan-A Transformation Assays

Cells (2×10^6) were nucleofected with 1.45 pmol of pcDNA6 ^{V600E}B-Raf, pcDNA6 AA-^{V600E}B-Raf alone, or in combination with pcDNA3.1puro myr-Akt3 or E40K-Akt3. Cells (4×10^4) per 50 μL were added to 96-well PolyHEMA-coated plates. After overnight recovery, 50 μL DMEM-supplemented with 10% FBS, 6 μg/mL puromycin, and/or 6 μg/mL blasticidin were added to give a final concentration of 3 μg/mL. After 3 d of selection, cell viability was quantified by MTS assay.

^{V600E}B-Raf Kinase Assay

Hemagglutinin-tagged pcDNA6 ^{V600E}B-Raf, S364A ^{V600E}B-Raf, S428A ^{V600E}B-Raf, AA-^{V600E}B-Raf (S364A and S428A), or Dead B-Raf (1.45 pmol) were nucleofected together with 1.45 pmol pcDNA 3.1myc-His myr-Akt3 into melan-a cells to phosphorylate ectopically expressed B-Raf at serine residues 364 and 428. Cell lysates were harvested 3 d later using lysis buffer containing 20 mmol/L Tris (pH 8.0), 2 mmol/L EDTA, 50 mmol/L β-glycerophosphate, 1 mmol/L activated sodium orthovanadate, 1% Triton X-100, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, and Protease Inhibitor Cocktail (Sigma). Various Hemagglutinin-tagged B-Raf proteins were immunoprecipitated from 150 μg of total lysates using 0.2 μg of antibody against Hemagglutinin, which was precoupled to Gamma Bind G sepharose (Amersham Pharmacia Biotech). Activity of immunoprecipitated B-Raf was measured as previously reported (42) using full-length Mek protein substrate (Santa Cruz Biotechnology). Samples were separated on duplicate NuPAGE gels (Invitrogen), with protein on one gel transferred onto polyvinylidene difluoride membrane (Western blot analysis) and other gel fixed in 10% acetic acid/50% methanol, dried, and then exposed to HyBlot CL autoradiography film (Denville) for ^{32}P Mek detection.

Phosphorylated B-Raf Analysis

Hemagglutinin-tagged B-Raf expressing plasmids [pcDNA6 wild-type B-Raf, ^{V600E}B-Raf, S364A ^{V600E}B-Raf, and AA-^{V600E}B-Raf (S364A, S428A)] were nucleofected separately or in combination with pcDNA 3.1myc-His myr-Akt3 into melan-a cells, seeded into PolyHEMA-coated plates and cell lysates harvested 3 d later. Various Hemagglutinin-tagged B-Raf proteins were immunoprecipitated from 20 to 40 μg of total lysates using 0.1 μg of antibody against Hemagglutinin using Gamma Bind G sepharose. Samples were separated on NuPAGE gels for Western blot analysis and probed with anti-Hemagglutinin and antiphosphorylated-Akt substrate monoclonal antibody (Cell Signaling). Western blot images were scanned and quantified using the Image Gauge 4.0 software (Fuji Photo Film Co.).

Animal Studies

siRNA (112.5 pmol for Akt3, C-Raf, and scrambled; 12.5 pmol for ^{V600E}B-Raf together with 100 pmol of scrambled siRNA; or 12.5 pmol for ^{V600E}B-Raf together with 100 pmol of Akt3 siRNA) was nucleofected into 1×10^6 UACC 903 cells. Thirty-six hours later, 1×10^6 UACC 903 cells in 0.2 mL of DMEM solution were injected s.c. into the left and right flanks of 4- to 6-wk-old nude mice. Dimensions of developing tumors were measured on alternate days up to day 17.5.

Statistical Analysis

One-way or Two-way ANOVA was used for groupwise comparisons, followed by the Tukey's or Bonferroni *post hoc* test. Results were considered significant at a P value of <0.05. Chou-Talalay method (Calcusyn software) was used for determining the combination Index (Biosoft; ref. 43).

Results

PI3k pathway inhibition increases MAPK activity in melanomas but not normal melanocytes. In melanomas, the PI3k pathway is activated through increased Akt3 expression and/or PTEN loss (7, 39). The MAPK pathway is primarily activated through mutation of B-Raf to a constitutively active V600E form (7, 21, 22, 24, 39, 44). Whether these pathways cooperate in early melanoma development remains an unanswered question. To investigate this possibility, activation of each pathway in melanocytes, having normal levels of Akt3 activity and wild-type B-Raf, was compared with UACC 903 melanoma cells having increased Akt3 activity as well as constitutively active ^{V600E}B-Raf, after growth factor starvation and stimulation. Densitometric analysis of Western blots quantified pErk and pAkt levels, which estimates MAP

and PI3K pathway activity, respectively, and were plotted as a continuum from 0 to 180 min after serum stimulation (Fig. 1*A* and *B*). Note, profiles represent best-fit lines showing minor perturbations due to graphical software. In starved melanocytes, activity levels of both pathways were initially low (Fig. 1*A*); however, upon growth factor stimulation, activity of both pathways increases transiently and declined over time to basal levels due to receptor desensitization, as reported for other model systems (34). In contrast, UACC 903 cells, which had significantly higher basal activity than melanocytes, showed an increase in pAkt accompanied by a corresponding decrease in MAPK pathway activity determined by measuring pErk (Fig. 1*B*; refs. 7, 14, 21). After serum stimulation, activity of both pathways in UACC 903 cells increased; however, as observed with other cell systems, growth factor stimulation led to transient increases in activity of both pathways that declined over time (34). This decrease was not observed after Akt inhibition using LY-294002; rather, pErk plateaued at high levels. Representative Western blots showing pErk and pAkt levels 30 and 60 min after growth factor or serum stimulation are shown in Fig. 1*C*. Although melanocytes showed no increase in pErk levels after inhibition of Akt using LY-294002, inhibition increased pErk levels for both UACC 903 and SK-MEL-24 cell lines (Fig. 1*C*). Different serum stimulation times that corresponded to the peak pAkt/Erk levels for each cell line were selected to easily observe changes in phosphorylation after agent manipulation (34). Thus, inhibition of PI3K pathway increased MAPK activity, suggesting inhibitory cross-regulation between these pathways in melanoma cells but not melanocytes.

Akt3 kinase regulates MAPK pathway activity. To establish whether Akt3 in the PI3K signaling cascade down-regulated MAPK pathway activity, siRNA targeting Akt3 was introduced into UACC 903 cells to inhibit protein expression, and cells were starved, then stimulated with serum followed by measurement of pAkt/Erk levels. Reducing Akt3 protein expression (activity) in UACC 903 cells caused a corresponding decrease in pAkt levels (Fig. 1*D*). In contrast, no change was observed in levels of pErk in melanocytes after siRNA-mediated knockdown of Akt3, similar to results observed after pharmacologic inhibition of Akt signaling in melanocytes (Fig. 1*C*). Significantly, decreased Akt3 activity caused a corresponding increase in MAPK activity observed by measuring pErk1/2 levels. Thus, Akt3 regulated the activity of the MAPK pathway.

Presence of ^{V600E}B-Raf and absence of constitutively activated Akt3 increased MAPK activity. The interactive roles of Akt3 and mutant ^{V600E}B-Raf in melanoma development were evaluated by measuring *in vitro* anchorage-independent growth of WM35 cells. Cells were derived from an early stage melanocytic lesion in the radial growth phase containing the (T1799A) B-Raf mutation (19). However, unlike UACC 903 cells, WM35 cells have low endogenous Akt3 activity due to presence of functional PTEN (7). Thus, WM35 cells are similar to ~90% of human nevi containing ^{V600E}B-Raf and having low Akt3 activity (6, 7, 14, 15, 44), making it a suitable model for evaluating the role played by Akt3 in melanocytic tumor progression. Densitometric analysis of Western blots showed pErk/Akt levels in WM35 cells after stimulation with growth factors after 24 h starvation (Fig. 2*A*). Upon growth factor stimulation, activity of both pathways increased transiently in WM35 cells and, over time, pAkt declined to basal levels due to receptor desensitization similar to that observed for melanocytes because both cell types contain PTEN (19, 34). In contrast, levels of pErk did not decline to near baseline levels; rather, pErk levels remained at near maximal amounts for duration of the experiment due to ^{V600E}B-Raf constitutively activating the downstream MAPK pathway (Figs. 1*A* and 2*A*). Thus, presence of ^{V600E}B-Raf and absence of constitutively activated Akt3 led to high MAPK activity, with potential to retard tumor progression.

Regulating MAPK pathway activity by targeting Akt3. Regulation of ^{V600E}B-Raf activity by Akt3 was examined using pharmacologic inhibition of Akt or expression of active myristoylated Akt3 in WM35 cells to examine effect on pErk/Akt levels. Although WM35 cells have low levels of pAkt compared with UACC 903 cells, inhibition of Akt using LY-294002 decreased levels of pAkt, causing a corresponding increase in pErk (Fig. 2*B*) similar to that observed for advanced melanoma cell lines (Fig. 1*C*). Because WM35 cells had levels of pAkt similar to those observed in normal melanocytes, constructs ectopically expressing constitutively active Akt3 were used to produce pAkt levels similar to those observed in more aggressive melanomas to confirm Akt3-mediated regulation of MAPK pathway activity. Western blot analysis showed increased pAkt accompanied by a corresponding decrease in pErk after ectopic expression of active myristoylated Akt3 but not dead Akt3 (control-producing inert protein; Fig. 2*B*). Thus, cross regulation between Akt3 and MAPK pathway activity occur with active Akt3.

^{V600E}B-Raf and Akt3 promote anchorage-independent growth. Effect of ^{V600E}B-Raf and Akt3 expression on melanocytic transformation was measured by regulating expression of each protein and quantifying anchorage-independent growth (a phenotypic measurement of cell transformation). WM35 cells were nucleofected with siRNA against B-Raf and seeded onto Poly-HEMA-coated 96-well plates. A significant decrease of viable WM35 cells was observed after nucleofection with B-Raf siRNA compared with a scrambled control ($P < 0.05$; One-way ANOVA; Fig. 2*C*). Western blot analysis confirmed B-Raf protein knockdown and corresponding decrease in downstream pErk (Fig. 2*C*). Next, myristoylated Akt3 having high activity or the E40K-Akt3 mutant having activity in the normal physiologic range was expressed in WM35 cells containing endogenous ^{V600E}B-Raf to determine whether it could enhance anchorage-independent growth (45). Akt3 expression significantly enhanced anchorage-independent growth compared with vector controls, which was directly proportional to level of pAkt ($P < 0.001$; One-way ANOVA; Fig. 2*D*). Expression of wild-type protein had lowest pAkt and least effect on anchorage-independent growth. To show that Akt3-mediated anchorage-independent growth of WM35 cells was not due to decreased apoptosis reported in advanced melanoma (7, 9), the downstream target of Akt3 regulating apoptosis, called PRAS40, was knocked down using siRNA while active Akt3 was expressed (9). Similar anchorage-independent growth was observed irrespective of levels of PRAS40 expression (Fig. 2*D*). Thus, deregulated apoptosis mediated by active Akt3 was not leading to enhanced anchorage-independent growth. This raised the possibility that Akt3 could be interacting with ^{V600E}B-Raf to regulate anchorage-independent growth because Akt has been reported to down-regulate B-Raf activity through direct phosphorylation (34–36). Thus, ^{V600E}B-Raf and Akt3 mediate anchorage-independent growth in the WM35 cell model and potentially cooperate in melanocyte transformation.

Akt3 cooperates with ^{V600E}B-Raf to transform melanocytes. Because Akt3 and ^{V600E}B-Raf independently regulated anchorage-independent growth of WM35 cells, effect of ectopic coexpression on melanocyte transformation was examined using melan-a mouse

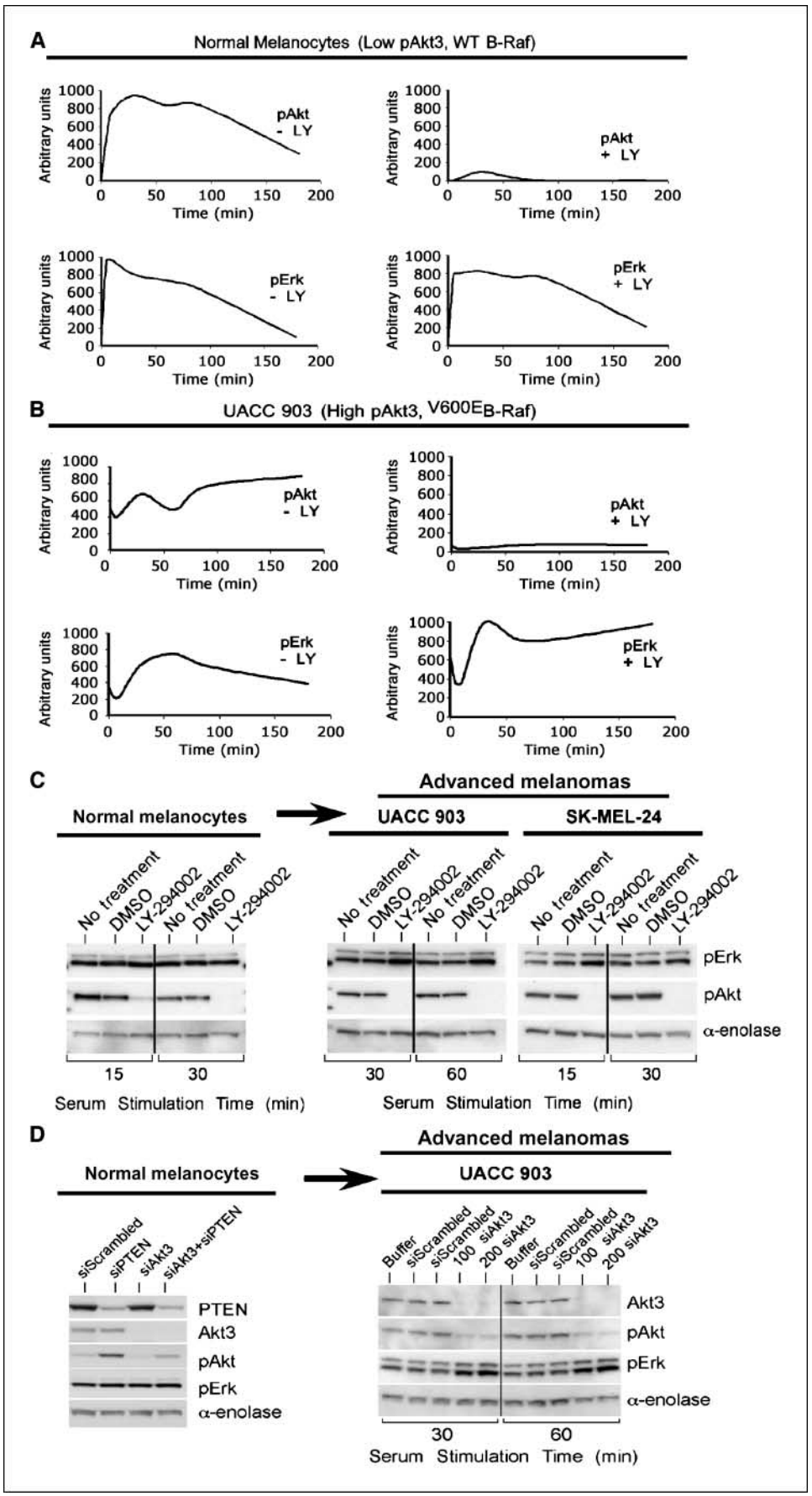

Figure 1. Crosstalk between the Akt3 and MAPK pathway occurs in melanoma cells but not normal melanocytes. Phosphorylated Akt and Erk levels of melanocytes (*A*) and UACC 903 (*B*) treated with or without LY-294002. Graphs represent densitometric scans of pAkt and pErk Western blots normalized to a α-enolase loading control. Cells were starved 24 h, treated with or without LY-294002 for 20 min, then serum stimulated before harvesting lysates at different time points for Western blot analysis. Analysis shows LY-294002 abrogates activation of Akt (pAkt) and consequently increases Erk activation (phosphorylation) in melanoma cells but not in human melanocytes. *C,* inhibition of Akt activation results in higher pErk levels in melanoma cell lines but not in melanocytes. Western blots are shown for pAkt and pErk levels in normal human melanocytes and two human metastatic melanoma cell lines, UACC 903 and SK-MEL-24. Cells were starved 24 h, then untreated or treated with DMSO or 50 mmol/L LY-294002 for 20 min, and then stimulated with growth factors or serum in medium and protein lysates harvested for Western blot analysis. The two metastatic melanoma cell lines showed evidence that LY-294002 treatment increased pErk levels, which did not occur in normal human melanocytes. *D,* inhibition of Akt3 increased pErk levels in melanoma cells. Normal melanocytes were transfected with scrambled siRNA (*siScrambled*), or siRNA targeting PTEN (*siPTEN*), or Akt3 (siAkt3) alone or in combination. After 48 h, cell lysates were analyzed for PTEN, Akt3, pAkt, and pErk levels. Inhibiting Akt3 protein expression had no effect on pErk levels. UACC 903 cells were transfected with buffer, scrambled siRNA, or Akt3-targeting siRNA, starved 24 h, then stimulated with medium-containing serum for 30 or 60 min, and protein lysates harvested for Western blot analysis. siRNA-mediated inhibition of Akt3 increased pErk levels compared with buffer or scrambled siRNA controls. α-enolase served as a control for protein loading.

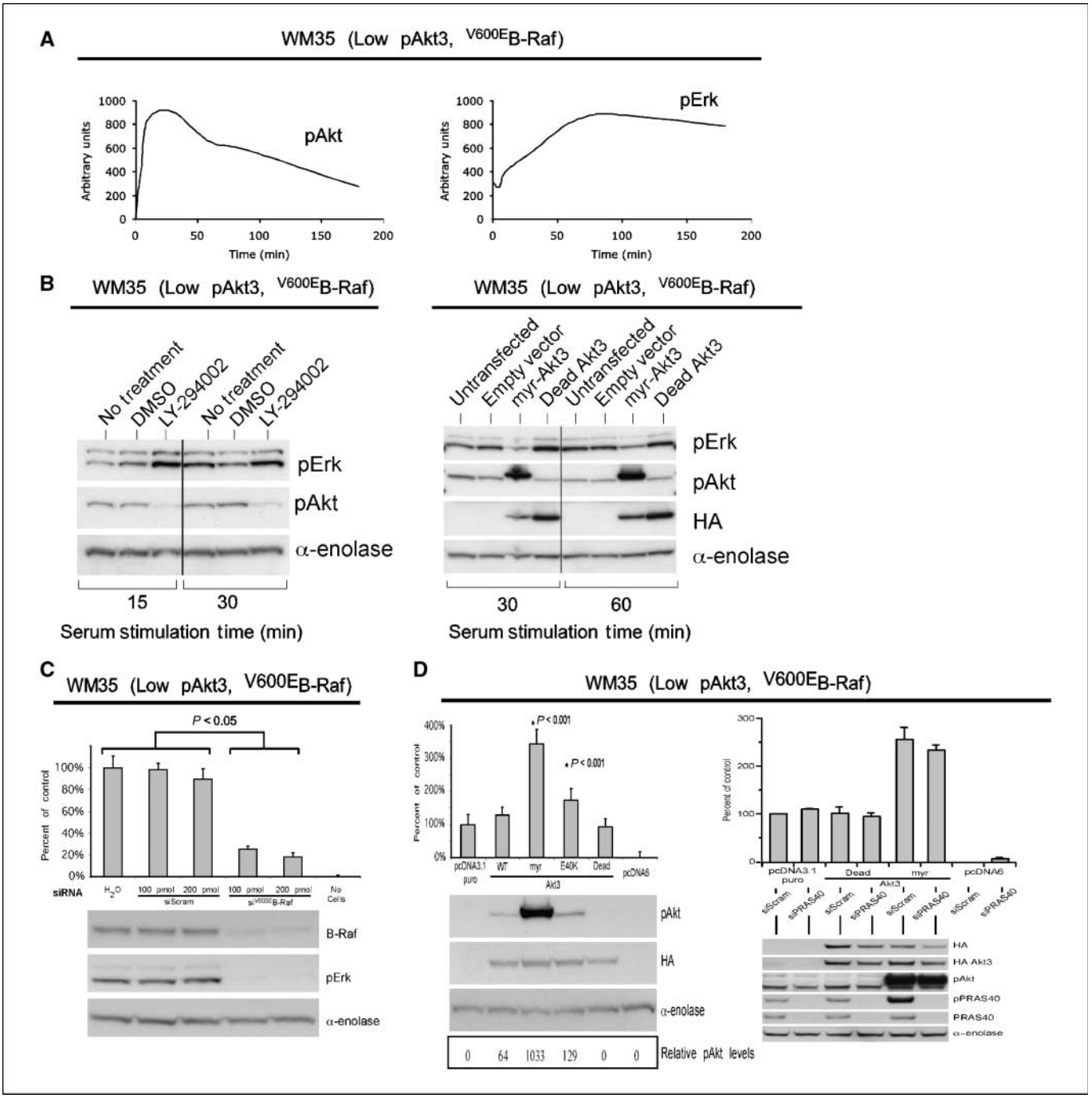

Figure 2. ^{V600E}B-Raf and Akt3 regulate anchorage-independent growth of early melanoma cells. *A,* WM35 cells have constitutive activation of MAPK pathway signaling due to presence of ^{V600E}B-Raf but normal Akt activity. Densitometric quantitation of pAkt and pErk levels from Western blots of the WM35 cell line after 24 h of serum starvation followed by stimulation shows constitutively high levels of pErk despite serum starvation, which is caused by ^{V600E}B-Raf activating the MAPK signaling cascade. In contrast, the Akt activation profile is similar to that observed for melanocytes. *B,* inhibition of pAkt in WM35 cells using LY-294002 resulted in higher pErk, whereas expression of active Akt3 decreased pErk levels. Treatment with LY-294002 led to a reduction in pAkt and a corresponding increase in pErk levels. Ectopic expression of active Akt3 decreased pErk levels. WM35 cells were nucleofected with Akt3 expressing constructs, and after 2 d of recovery, cells were starved for 24 h, and then stimulated with serum-containing medium and protein lysates collected for Western blot analysis. Ectopic myristoylated-Akt3 expression led to decreased pErk levels. *C,* inhibition of B-Raf decreases anchorage-independent growth of early melanoma cells. WM35 cells were nucleofected with siRNA targeting ^{V600E}B-Raf (100 and 200 pmol), scrambled siRNA, or a water control. Cells were then plated onto PolyHEMA-coated 96-well plates to measure the effect on anchorage-independent growth. MTS assay, 3 d later, was used to quantify viable cell number. Western blot showed efficient knockdown of B-Raf protein expression and decreased pErk levels. *D,* ectopic expression of active Akt3 enhanced anchorage-independent growth. Viability of WM35 cells nucleofected with Akt3 is shown along with Western blot analysis of cell lysates. WM35 cells were nucleofected with various Akt3 expressing constructs: empty vector, wt-Akt3, Dead-Akt3, and active Akt3; myristoylated-Akt3 (*myr-Akt3*) or E40K-Akt3 alone or in combination with a siRNA targeting PRAS40 protein. After nucleofection, cells were plated directly onto PolyHEMA-coated 96-well plates, and after 3 d of growth in selection medium, an MTS assay was used to quantify cell viability. Myr-Akt3 significantly increased cell growth compared with less active E40K-Akt3 that had a more modest effect. Changes in cell growth corresponded directly to pAkt level, which is shown by Western blot analysis and quantified by densitometry. Knocking down PRAS40 had no significant effect on cell viability indicating that deregulated apoptosis mediated by active Akt3 was not leading to enhanced anchorage independent growth. Hemagglutinin-probed Western blot shows equal ectopic expression of the different Akt3 proteins. α-enolase served as a control for protein loading.

melanocytes to assess transformation by anchorage-independent growth. This model was chosen because melan-a cells undergo transformation after low level ^{V600E}B-Raf expression (46, 47). Ectopically expressed ^{V600E}B-Raf or active-Akt3 alone, promoted anchorage-independent growth compared with empty vector ($P < 0.001$; Fig. 3*A*). Numerous groups have reported the oncogenic properties of myr-Akt3 (48). However, a limitation is that the activity resulting from myr-Akt3 is very high, which could be an explanation for cell transformation. Therefore, we incorporated the E40K Akt3 mutant that leads to more moderate physiologic pAkt levels similar to those present in more aggressive melanoma cells. Coexpression of ^{V600E}B-Raf with active E40K- or myr-Akt3 led to statistically significant enhanced anchorage-independent growth compared with single genes (*lanes 5* versus *2* or *3*, $P < 0.05$; *lanes 6* versus *2* or *4*, $P < 0.001$; Fig. 3*A*). Western blot analysis of cell lysates showed that active myr-Akt3 reduced levels of pErk accompanying ^{V600E}B-Raf expression (Fig. 3*A*). A similar but less significant effect was seen after E40K Akt3 coexpression with ^{V600E}B-Raf (Fig. 3*A*). These results suggest that active Akt3 and ^{V600E}B-Raf cooperate in melanocyte transformation, promoting anchorage-independent growth.

Akt3 kinase phosphorylates ^{V600E}B-Raf to lower *in vitro* activity. To determine whether phosphorylation mediated by Akt3-regulated ^{V600E}B-Raf activity, serine residues 364 and/or 428 in ^{V600E}B-Raf were converted to alanine. Plasmids expressing these B-Raf constructs were conucleofected with myr-Akt3 into melan-a cells to promote phosphorylation of ectopically expressed B-Raf proteins on Ser^{364} and Ser^{428}. ^{V600E}B-Raf constructs containing alanine at residues 364 and/or 428 could not be phosphorylated by Akt3. Various Hemagglutinin-tagged B-Raf proteins were then immunoprecipitated, and activity was measured using an *in vitro* kinase assay with Mek as the substrate (Fig. 3*B*). AA-^{V600E}B-Raf (S364A and S428A) exhibited highest level of activity compared with ^{V600E}B-Raf, ^{V600E}B-Raf (S364A), or ^{V600E}B-Raf (S428A; $P < 0.05$; Fig. 3*B*), demonstrating that direct phosphorylation of ^{V600E}B-Raf protein by Akt3 decreased its activity. Thus, phosphorylation of ^{V600E}B-Raf has significant potential to decrease activity of the mutant protein.

Active Akt3 phosphorylates ^{V600E}B-Raf in melanocytic cells. To show that Akt3 phosphorylates mutant ^{V600E}B-Raf in melanocytic cells, melan-a melanocytes were transfected with Hemagglutinin-tagged ^{V600E}B-Raf, ^{V600E}B-Raf (S364A), or AA-^{V600E}B-Raf (S364A and S428A) alone or with myr-Akt3. Western blot analysis using an antibody recognizing consensus sequences typically phosphorylated by Akt was used to quantify levels of phosphorylation of immunoprecipitated Hemagglutinin-tagged B-Raf from cells (Fig. 3*C*). ^{V600E}B-Raf (S428A) was not used because the antibody does not recognize this site. Increased phosphorylation of ^{V600E}B-Raf occurred when coexpressed with active Akt3 ($P < 0.01$; Fig. 3*C*). Mutation of Ser^{364} to alanine [^{V600E}B-Raf (S364A)] decreased pB-Raf levels ($P < 0.01$; Fig. 3*C*). Furthermore, mutation of both Ser^{364} and Ser^{428} to alanines [AA-^{V600E}B-Raf (S364A and S428A)] completely blocked phosphorylation by Akt3 ($P < 0.01$; Fig. 3*C*). Thus, active Akt3 is capable of phosphorylating ^{V600E}B-Raf in melanocytic cells.

Phosphorylation of ^{V600E}B-Raf decreases MAPK pathway signaling, overcoming inhibitory growth effects associated with high activity. Effect of phosphorylated versus unphosphorylated ^{V600E}B-Raf on melanocytic transformation was examined by expressing ^{V600E}B-Raf or AA-^{V600E}B-Raf (S364A and S428A) alone or with active Akt3 in melan-a cells and measuring anchorage-independent growth. Compared with ^{V600E}B-Raf expression alone, AA-^{V600E}B-Raf (S364A and S428A) protein significantly decreased cell proliferation ($P < 0.001$; Fig. 4*A*). Similarly, coexpression of AA-^{V600E}B-Raf (S364A and S428A) with myristoylated or E40K Akt3 also significantly reduced proliferation when compared with cells expressing ^{V600E}B-Raf or Akt3 (Fig. 4*A*; $P < 0.001$). Western blot analysis of cell lysates showed AA-^{V600E}B-Raf (S364A and S428A) expression accompanied by higher MAPK pathway activity than what occurred with ^{V600E}B-Raf (Fig. 4*B*). Furthermore, coexpression of AA-^{V600E}B-Raf (S364A and S428A) with myristoylated-Akt3 did not lower pErk levels as observed when ^{V600E}B-Raf and myristoylated Akt3 were coexpressed (Fig. 4*B*). Induction of cyclin D1 was also impaired after AA-^{V600E}B-Raf (S364A and S428A) expression (Fig. 4*B*). Thus, phosphorylation of ^{V600E}B-Raf by Akt3 decreased MAPK signaling, overcoming growth inhibitory effects associated with high pathway activity.

Targeting ^{V600E}B-Raf and Akt3 in melanomas cooperatively inhibits tumorigenesis. To evaluate the therapeutic potential of simultaneously inhibiting Akt3 and mutant ^{V600E}B-Raf in melanoma tumorigenesis, a siRNA was used to inhibit protein expression. Effects on tumor development was studied by nucleofecting siRNA against each target into UACC 903 cells, allowing 1.5 days of recovery in culture followed by s.c. injection of 1 million cells into nude mice. Reduced expression of each protein was observed for as long as 9.5 days after nucleofection in cultured cells and in tumors of nude mice (data not shown; ref. 25). Size of developing tumors was measured using calipers every other day until day 17. Initially, siRNA against Akt3 and B-Raf was titered to establish optimal concentration inhibiting tumorigenesis to a similar extent. One hundred pmol of Akt3 siRNA was found to inhibit tumorigenesis similar to that occurring with 12.5 pmol of siRNA against B-Raf (Fig. 5*A*). In comparison to controls (buffer, scrambled siRNA, and C-Raf siRNA), siRNA against Akt3 or ^{V600E}B-Raf significantly decreased the tumorigenic potential of the UACC 903 cells (Fig. 5*A*). An even more dramatic reduction in tumorigenesis was observed after simultaneous inhibition of both Akt3 and B-Raf. Under these conditions, cooperatively acting tumor inhibition was observed ($P < 0.001$; Two-way ANOVA; Fig. 5*A*). Tumors removed from mice 9.5 days after siRNA nucleofection were found to have reduced Akt3 (Fig. 5*B*) or ^{V600E}B-Raf (Fig. 5*C*) protein expression, demonstrating effective knockdown of these proteins. Thus, targeting both Akt3 and B-Raf led to cooperatively acting tumor inhibition.

Simultaneously targeting ^{V600E}B-Raf and Akt3 inhibits anchorage-independent growth. Because targeting Akt3 and ^{V600E}B-Raf led to cooperative tumor inhibition, mechanistic basis for inhibition was examined next. siRNA targeting Akt3 and/or ^{V600E}B-Raf was nucleofected into UACC 903 cells, and its effect on *in vitro* anchorage-independent growth was examined. Simultaneous siRNA-mediated inhibition of Akt3 and B-Raf dramatically inhibited growth of cells compared with either Akt3 or B-Raf (Fig. 6*A*, *lanes 2, 4*, and *7* and *lanes 2, 5*, and *8*; ANOVA, $P < 0.001$; ref. 49). To determine whether inhibition of anchorage-independent growth was additive or synergistic after siRNA-mediated inhibition of Akt3 and ^{V600E}B-Raf, the Chou-Talalay method for determining the combination index using Calcusyn software was undertaken (43). Using this approach, combination index values of <0.9 are synergistic, of >1.1 are antagonistic, and values of 0.9 to 1.1 are nearly additive. Experimentally, Akt3 siRNA was kept at a constant dose of 200 pmol, whereas the concentration of B-Raf siRNA was titrated at doses of 3, 6, and 12 pmol. The resulting combination

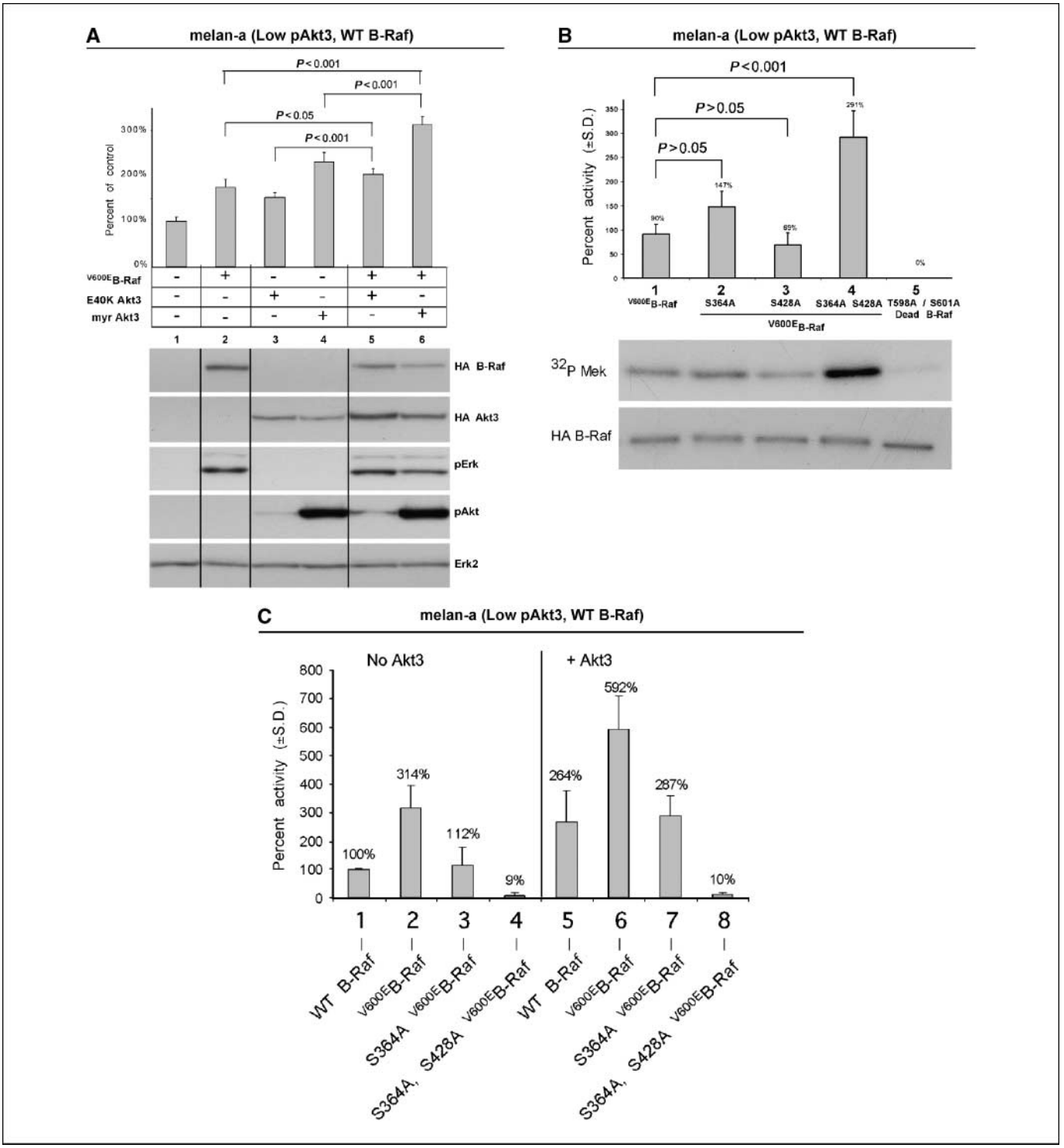

Figure 3. V600EB-Raf and Akt3 cooperate in melanocyte transformation. *A,* Melan-a transformation, after ectopic V600EB-Raf and Akt3 expression, was quantified by measuring effect on anchorage-independent growth. Melan-a cells were nucleofected with various combinations of V600EB-Raf and Akt3 expression plasmids (resistant to blasticidin and puromycin, respectively) and then directly plated onto PolyHEMA-coated 96-well plates. Transfected cells were selected for blasticidin and puromycin resistance for 3 d followed by MTS assay to quantify cell viability. Results of a representative MTS assay are shown indicating effects of V600EB-Raf and Akt3 expression on anchorage-independent growth. Nucleofections of either V600EB-Raf or myr-Akt3 led to increased cell viability; however, coexpression of both genes resulted in a more statistically significant effect. Western blots indicate changes in signaling pathways after ectopic expression of B-Raf and Akt3. *Columns,* mean; *bars,* SD. *B, in vitro* B-Raf kinase assay showing activity of V600EB-Raf compared with protein in which phosphorylation sites are converted to alanine S364A and S428A. Top blot shows autoradiography film of 32P-labeled Mek substrate, whereas lower shows a Western blot quantifying the amount of immunoprecipitated B-Raf protein used for the kinase assay. The graph represents average densitometry analysis (±SD) of three independent experiments, where values indicate level of labeled Mek normalized to the amount of Hemagglutinin tagged B-Raf, relative to Dead B-Raf set as the background control. *C,* Akt3 phosphorylates V600EB-Raf on Ser364 and Ser428. Quantitation of B-Raf phosphorylation using densitometric scans of Western blot that were normalized to total immunoprecipitated Hemagglutinin tagged B-Raf. Both wild-type and V600EB-Raf were phosphorylated by Akt3. Conversion of Ser364 to alanine reduced levels of V600EB-Raf phosphorylation, whereas mutation of both Ser364 and Ser428 completely abrogated phosphorylation by Akt3. *Columns,* mean average of three independent experiments; *bars,* SD.

index for each combination was calculated to be 0.909, 1.023, and 1.107, for 3, 6, and 12 pmol of B-Raf siRNA, respectively combined with 200 pmol of Akt3 siRNA, which suggest nearly additive cooperation. Thus, simultaneously targeting ^{V600E}B-Raf and Akt3 inhibits anchorage-independent growth in a cooperatively additive manner.

Simultaneously targeting both Akt3 and ^{V600E}B-Raf enhances growth inhibition and apoptotic effects on signaling cascades. Mechanistic basis for inhibition of anchorage-independent growth after ^{V600E}B-Raf and Akt3 targeting was examined using Western blotting to measure levels of protein expression involved in proliferation (cyclin D1) and apoptosis (cleaved caspase-3). Control UACC 903 cells had high cyclin D1 and low cleaved caspase-3 levels (Fig. 6*B, lane 1*). In contrast, siRNA-mediated inhibition of Akt3 increased cleaved caspase-3 levels, indicating increased cellular apoptosis (Fig. 6*B, lane 2*). B-Raf siRNA did not significantly alter levels of cleaved caspase 3 but did significantly decreased cyclin D1 levels with a corresponding increase in p27 (Fig. 6*B, lanes 3–5*). Significantly, inhibition of both Akt3 and mutant ^{V600E}B-Raf dramatically increased levels of cleaved caspase 3, indicative of high apoptosis compared with cells in which Akt3 alone had been inhibited (Fig. 6*B, lanes 6, 7*, and *8* versus *lane 2*). Furthermore, the combination approach led to more dramatic decrease in cyclin D1 than observed when ^{V600E}B-Raf was inhibited alone. Thus, simultaneously targeting both Akt3 and ^{V600E}B-Raf has a more dramatic effect on the signaling cascades of these pathways than inhibiting each individually.

Discussion

Nevi or moles are benign proliferations of melanocytes that initially proliferated and then stopped growing (30). Approximately 90% harbor activating B-Raf mutations but never progress into melanomas (12, 14, 15). This is caused by cellular senescence associated with high MAPK pathway activity due to presence of ^{V600E}B-Raf, which is linked to induction of cdk kinase inhibitors, such as p21^{Cip1}, p16^{Ink4a}, and p27^{Kip1}, functioning as a defense mechanism of normal cells to overcome oncogene activation

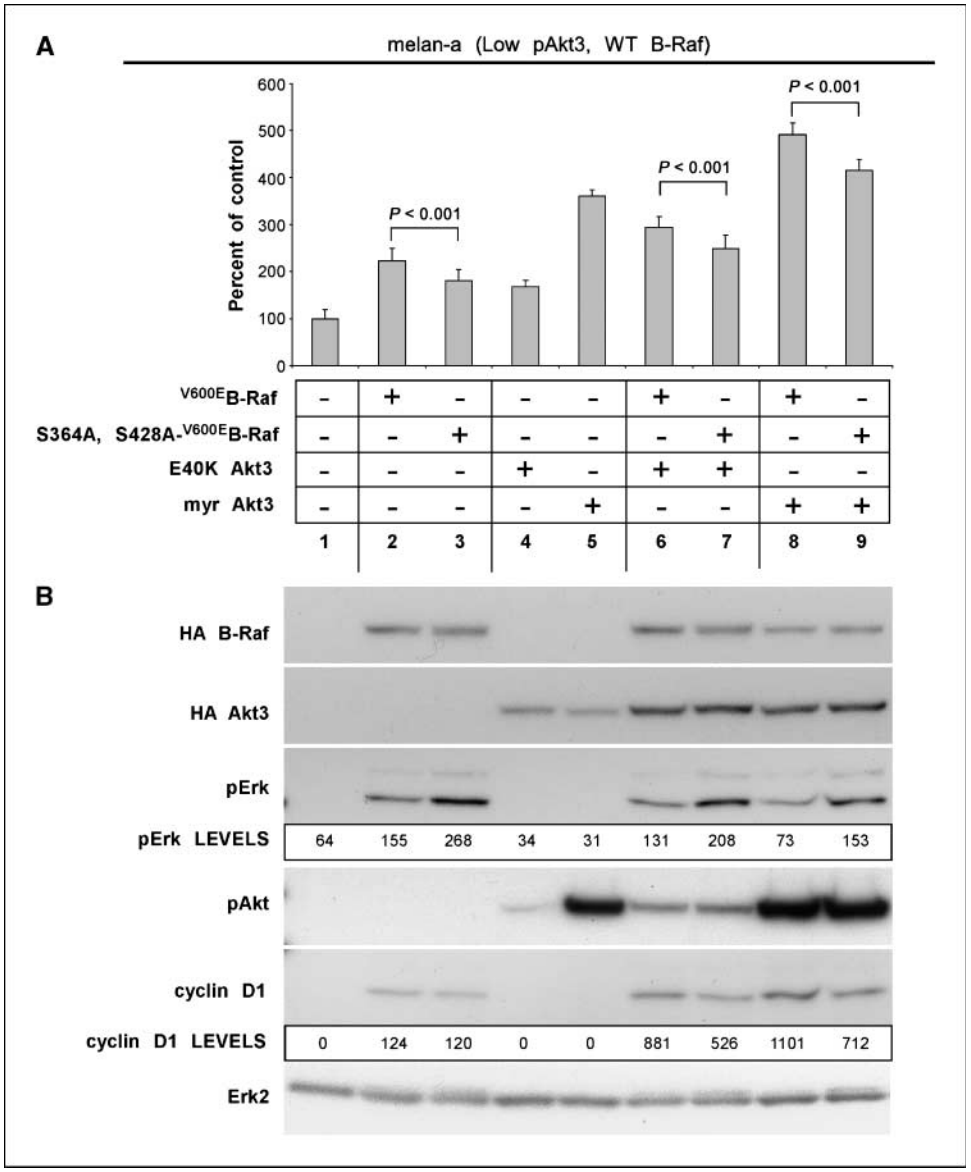

Figure 4. ^{V600E}B-Raf and Akt3-mediated transformation requires Akt3 phosphorylation of ^{V600E}B-Raf. *A,* representative graph showing anchorage-independent growth mediated by ^{V600E}B-Raf and Akt3 coexpression. Melan-a cells were nucleofected with different combinations of S364A/S428A ^{V600E}B-Raf (AA-^{V600E}B-Raf) or ^{V600E}B-Raf and Akt3-expressing plasmids, and plated onto PolyHEMA-coated 96-well plates. Transfected cells were then selected for 3 d for purimycin resistance followed by MTS assay to measure cell viability. Conversion of the two serine phosphorylation sites on ^{V600E}B-Raf to alanines (S364A/S428A) led to significantly decreased anchorage-independent growth mediated by ^{V600E}B-Raf and Akt3 expression; *columns,* mean of four independent experiments; *bars,* SD. *B,* Western blots showing changes in signaling pathways after ectopic expression of B-Raf and Akt3. Increased cyclin D1 levels are associated with coexpression of ^{V600E}B-Raf and Akt3.

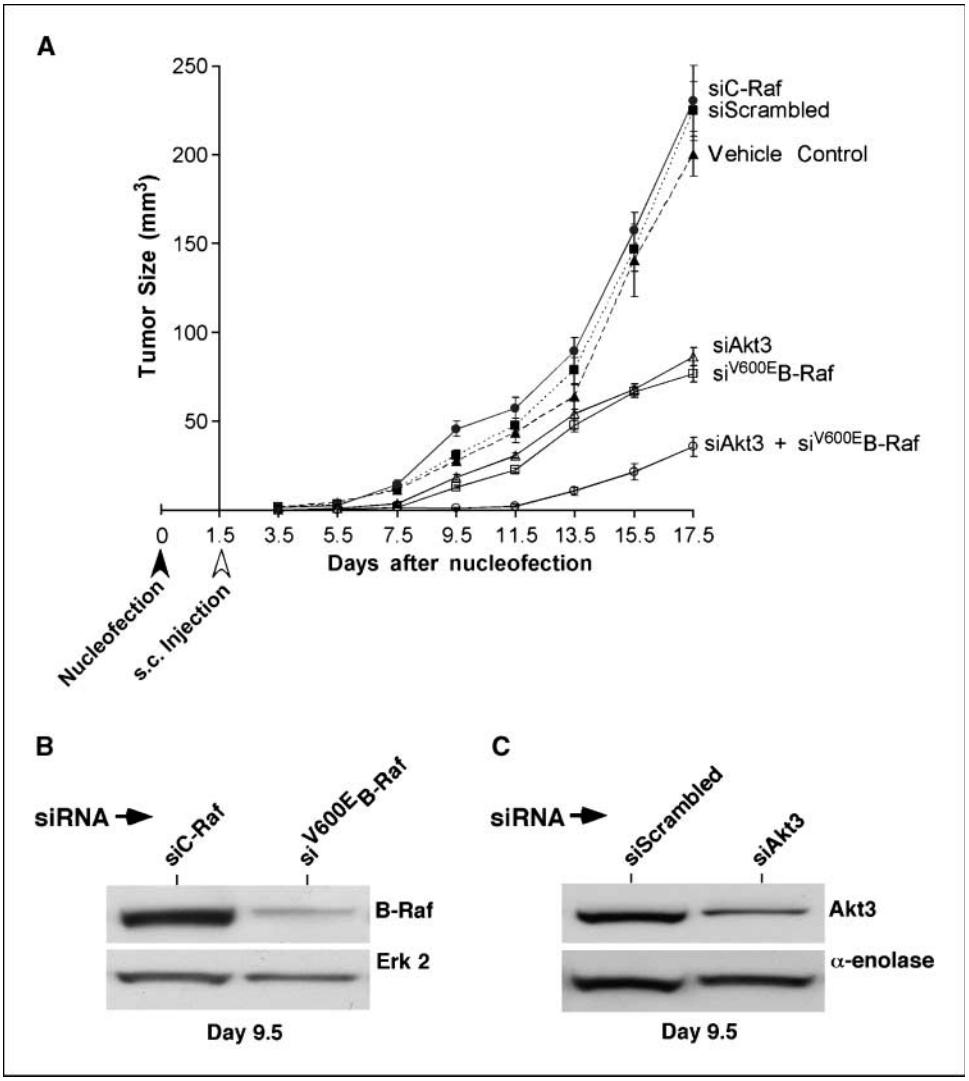

Figure 5. Targeting ^{V600E}B-Raf and Akt3 dramatically retards melanoma tumorigenesis. *A,* nucleofection of siRNA against both B-Raf and Akt3 into UACC 903 cells led to a statistically significant reduction of tumor development in animals. The cells were nucleofected with siRNA, allowed to recover in culture for 1.5 d, and then injected s.c. into nude mice. Tumor size was measured on alternate days up to day 17.5. Inhibition of Akt3 or B-Raf alone led to a significant reduction in growth compared with controls; however, targeting both proteins led to an even more dramatic reduction compared with targeting Akt3 or B-Raf individually. *Points,* mean; *bars,* SE. *B*, Western blot of UACC 903 tumors isolated from mice 8 d after nucleofection with siRNA targeting ^{V600E}B-Raf. siRNA against B-Raf significantly reduced level of B-Raf protein compared with control cells nucleofected with siRNA to C-Raf. *C*, Western blot of UACC 903 tumors isolated from mice 8 d after nucleofection with siRNA targeting Akt3. Akt3 protein level was significantly reduced with siRNA targeting Akt3 compared with cells nucleofected with a scrambled siRNA control.

(26, 28, 29). In contrast, moderate levels of MAPK pathway activation promote cell cycle progression and cancer development (27). Therefore, cells containing ^{V600E}B-Raf require additional genetic changes for senescent melanocytic cells to reenter the cell cycle (31).

Identification of proteins cooperating with ^{V600E}B-Raf in melanocytic transformation are being characterized by studying nevi development using transgenic zebrafish (30). Prolonged hyperactivation of MAPK signaling in zebrafish melanocytes mediated by ^{V600E}B-Raf initially promote cellular growth and development of structures resembling nevi followed by a senescent phase, a situation comparable with that occurring with most human moles (30). Zebrafish nevi did not develop into melanomas until p53 was deleted, suggesting deregulation of multiple pathways for melanocytic tumor progression (30). Thus, genes such as *Akt3* with prominent roles in the development of ~70% of melanomas likely cooperate with ^{V600E}B-Raf in melanocytic transformation (7, 9, 39, 44, 50).

Akt had been shown to regulate B-Raf activity (34, 35); however, it was unknown whether Akt3 would phosphorylate mutant ^{V600E}B-Raf and, if so, whether this process played any role in melanoma development. In this report, using a combination of pharmacologic and genetic approaches, Akt3 was shown to down-regulate ^{V600E}B-Raf-mediated activation of MAPK pathway signaling to promote tumor progression. Ectopic expression of active Akt3 in early melanoma cells, phenotypically resembling nevi cells by containing ^{V600E}B-Raf with low endogenous Akt3 activity, increased anchorage-independent growth. Furthermore, expression of both proteins in melanocytes promoted anchorage-independent growth, demonstrating that ^{V600E}B-Raf and active Akt3 act cooperatively together to promote melanocyte transformation; thereby, confirming the importance of these proteins in early melanoma development. Mechanistically, Akt3 was shown to directly phosphorylate B-Raf on serine residues 364 and 428 to regulate activity of mutant ^{V600E}B-Raf protein to promote melanocyte transformation (41). This second role for Akt in melanoma development was confirmed by inhibiting the downstream target of Akt3-regulating apoptosis, called PRAS40 (9). Constitutively active Akt3 was expressed to activate the signaling cascade, whereas siRNA-mediated knockdown was used to inhibit PRAS40 expression/activity. Because similar anchorage-independent growth was observed irrespective of levels of PRAS40 expression, deregulated apoptosis mediated by

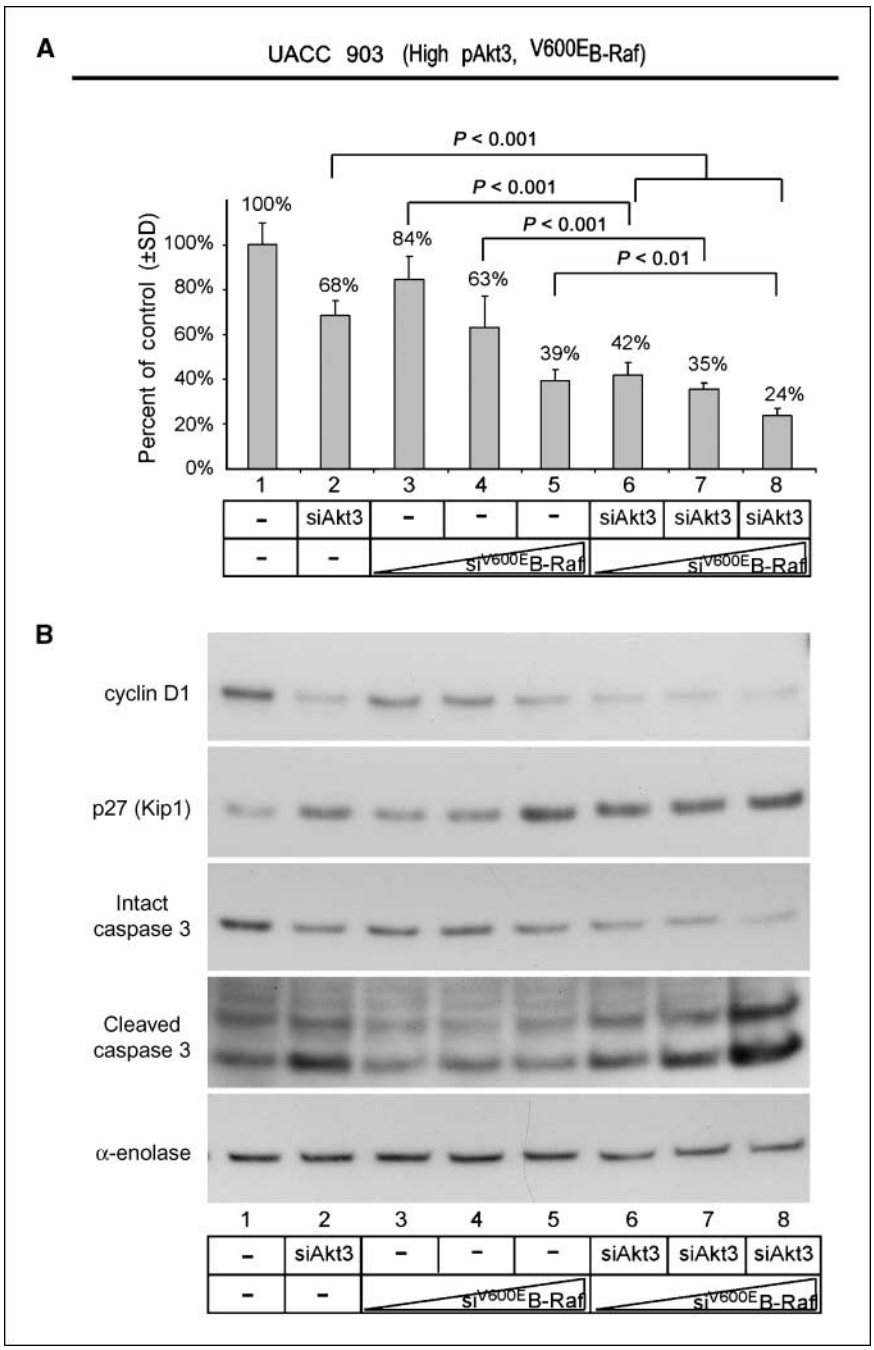

Figure 6. Simultaneously targeting ^{V600E}B-Raf and Akt3 inhibits *in vitro* melanoma growth in an additively cooperating manner. *A, in vitro* anchorage-independent growth of UACC 903 cells nucleofected with siRNA targeting ^{V600E}B-Raf and Akt3 in cell culture medium containing 0% serum. UACC 903 cells nucleofected with siRNA were plated onto PolyHEMA-coated 96-well plates, allowed to grow for 3 d in DMEM containing no serum and cell viability quantified by MTS assay. UACC 903 cells nucleofected with no siRNA (*lane 1*) was set as 100% and others compared with this value. Targeting both B-Raf and Akt3 resulted in statistically significant inhibition of growth compared with cells in which B-Raf or Akt3 had been targeted individually. *B,* Western blots showing effects of targeting ^{V600E}B-Raf and Akt3 on cyclin D1, cleaved caspase-3, and p27^{Kip1} protein levels. siRNA-mediated inhibition of ^{V600E}B-Raf and Akt3 led to significant reductions in cyclin D1 and corresponding increases in cleaved caspase3 and p27^{Kip1} protein levels compared with cells in which proteins had been targeted individually.

active Akt3 was not leading to enhanced anchorage-independent growth. Thus, a second important role has been identified for Akt3 in melanoma development.

Melan-a mouse melanocytes used as a model of transformation are considered to be a normal mouse melanocyte cell line and is an accepted model for studying ^{V600E}B-Raf-mediated transformation (47). However, it lacks p16^{INK4a} as well as alternative reading frame (ARF) protein expression. Data presented in this study show that melan-a transformation mediated by ^{V600E}B-Raf expression in a p16^{INK4a}/ARF null background requires expression of active Akt3 to promote anchorage-independent growth. This suggests that not only was loss of p16^{INK4a}/ARF important in bypassing ^{V600E}B-Raf induced senescence, but Akt3 activation was required to phosphorylate ^{V600E}B-Raf to modulate the level of MAPK activity.

The therapeutic potential of simultaneously targeting Akt3 and ^{V600E}B-Raf is also shown in this study. Tumor inhibition was most significant in animals containing cells in which both proteins had been targeted. *In vitro* studies confirmed the effects of the combination approach, demonstrating that targeting both led to cooperatively acting inhibition significantly increasing cellular apoptosis and decreasing proliferation. Thus, therapeutics targeting both pathways in human patients is predicted to be more effective than targeting each individually.

In conclusion, Akt3 has been shown to cooperate with ^{V600E}B-Raf in promoting melanoma development by phosphorylating the

mutant protein to decrease its activity to levels that promote rather than inhibit melanocytic cell growth. Furthermore, simultaneously targeting both Akt3 and ^{V600E}B-Raf was more effective at inhibiting melanomas than inhibiting each individually, which suggest that therapies for human patients should simultaneously target these signaling pathways for maximal clinical efficacy.

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Akt3 and Mutant ^{V600E}B-Raf Cooperate to Promote Early Melanoma Development

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