

ORIGINAL ARTICLE

B-RAF and PI-3 kinase signaling protect melanoma cells from anoikis

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A hallmark feature of cancer is resistance to anoikis, apoptosis induced when cells either lose contact with or encounter an inappropriate extracellular matrix. Melanoma is inherently associated with a high degree of resistance to apoptosis. Mutations in B-RAF are prevalent in melanoma and promote constitutive MEK–ERK1/2 signaling and cell transformation. Acquisition of B-RAF mutations correlates with vertical phase growth when melanoma cells invade into the dermis, a collagen-rich environment that also contains fibronectin matrix. In addition, alterations in phosphoinositide-3 kinase (PI-3 kinase) signaling that lead to activation of AKT are detected in advanced melanomas. Here we show that knockdown of B-RAF expression by siRNA or pharmacological inhibition of MEK rendered melanoma cells susceptible to anoikis. Furthermore, adhesion to fibronectin but not collagen protected melanoma cells from anoikis through a PI-3 kinase-dependent pathway. Therefore, melanoma cells require either B-RAF or PI-3 kinase activation for protection from anoikis. Notably, AKT signaling in melanoma cells is substrate specific. These findings demonstrate that melanoma cells utilize multiple signaling pathways to provide resistance to apoptosis.

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Introduction

Since the late 1950s, the incidence of melanoma has risen 10-fold (Bataille, 2003) and it is the fifth and seventh most commonly diagnosed cancer among American men and women, respectively (Gill and Celebi, 2005). Treatment exists for melanoma if it is detected early; however, once it metastasizes, survival rates decrease (Koh, 1991; Alonso *et al.*, 2004). Further study is needed to determine the genetic alterations in melanoma, and how they contribute to malignant properties.

B-RAF, a regulator of the MEK–ERK1/2 pathway, has been shown to be mutated in over two-thirds of human melanoma (Davies *et al.*, 2002). The most common mutation results in a glutamic acid substitution in place of valine at residue 600 in the B-RAF activation loop. Although B-RAF activation alone is not sufficient to transform a melanocyte, as B-RAF mutations are also found in melanocytic nevi (Pollock *et al.*, 2003), the constitutive MEK–ERK1/2 signaling resulting from the B-RAF^{V600E} mutation has been shown to regulate G1 cell cycle events in primary human melanocytes (Bhatt *et al.*, 2005) and to induce proliferation and transformation in immortalized murine melanocytes (Wellbrock *et al.*, 2004). The role of B-RAF in protection from apoptosis in melanoma cells is less clear. Hingorani *et al.* (2003) and Karasarides *et al.* (2004) have independently shown that suppression of B-RAF leads to increased apoptosis, whereas Sumimoto *et al.* (2004) found that B-RAF is not necessary for cell survival. Expression of phosphatase and tensin homolog (PTEN), a dual specificity lipid and protein phosphatase, is also frequently altered in melanoma. Approximately, 10% of melanomas have defects in PTEN, whereas in melanoma cell lines this percentage is increased to 30–40% (Guldberg *et al.*, 1997; Tsao *et al.*, 1998). Deficiency in PTEN leads to activation of AKT1/2/3, a family of kinases that play a major role in cell survival through effects on proteins including Bcl-X_L/Bcl-2-associated death promoter (Bad), forkhead transcription factor 1, NF- κ B, MDM2 and CREB (Datta *et al.*, 1997; del Peso *et al.*, 1997; Brunet *et al.*, 1999; Kane *et al.*, 1999; Wang *et al.*, 1999; Mayo and Donner, 2001). Thus, loss or reduction in PTEN expression provides tumor cells with a survival advantage. Additionally, AKT3 is frequently over-expressed and constitutively active in melanoma (Stahl *et al.*, 2004).

Melanocytes and radial growth phase melanomas reside in the epidermis or papillary dermis. A poor prognosis in melanoma is associated with the vertical depth of invasion into the dermis, a collagen I-rich layer containing fibronectin deposits. In order for cells to progress to the vertical growth phase and subsequent metastatic phase, they need to acquire resistance to apoptosis. One form of apoptosis, anoikis, occurs when cells either lose contact with or encounter an inappropriate extracellular matrix environment (Frisch and Francis, 1994). Cells sense their environment through interactions between distinct integrins and extracellular matrix proteins. Integrin engagement can

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lead to the activation of various survival pathways including RAF–MEK–ERK1/2 and PI-3 kinase–AKT, and both of these pathways protect epithelial cells from anoikis (Khwaja *et al.*, 1997; Davies *et al.*, 1998; Le Gall *et al.*, 2000; Fukazawa *et al.*, 2002); however, their roles in melanoma resistance to anoikis have not been defined.

As the conversion from radial to vertical growth phases is associated with gain of B-RAF mutation (Dong *et al.*, 2003) and PI-3 kinase signaling has been associated with protection from anoikis (Khwaja *et al.*, 1997), we tested the role of these signaling proteins and their downstream pathways in protecting melanoma cells from anoikis. We show that B-RAF^{V600E}–MEK and PI-3 kinase signaling protect melanoma cells from anoikis. Furthermore, we provide evidence that activation of AKT acts as an environmental sensor being activated upon attachment to fibronectin but not type I collagen. These results demonstrate that distinct signaling pathways can confer resistance to apoptosis in human melanoma cells.

Results

Primary human melanocytes are susceptible to anoikis, but melanoma cells harboring B-RAF^{V600E} are resistant

We initially analysed primary normal human epidermal melanocytes (NHEM) and a panel of human melanoma cell lines for susceptibility to anoikis. Serum-starved NHEM were allowed to adhere to fibronectin via integrins or to attach to poly-L-lysine, a condition in which integrins are not engaged. Apoptosis was detected via TdT-mediated dUTP nick-end labeling (TUNEL) staining. Significant numbers of apoptotic cells were observed only when NHEM were replated on poly-L-lysine in the absence of growth factors (Figure 1a and b). On average, 35% of NHEM undergo apoptosis in these conditions. Normal human epidermal melanocytes were protected from apoptosis by adhesion to fibronectin, addition of growth factors (Figure 1b), or adhesion to laminin 5 (data not shown).

To determine the effect of loss of adhesion on apoptosis in human melanoma cell lines that are characteristic of the different stages of melanoma progression, we utilized three cell lines: Sbc12, WM793 and SK-MEL-28. Sbc12 cells are characterized as a radial growth phase cell line and are wild type at the B-RAF locus; the vertical growth phase cell line WM793 and the metastatic cell line SK-MEL-28 both harbor the B-RAF^{V600E} mutation. In experiments similar to the above, we measured apoptosis by TUNEL staining following 48 h of plating on either poly-L-lysine or fibronectin. Sbc12 cells displayed a dramatic increase in apoptosis when they were plated on poly-L-lysine as compared to fibronectin (Figure 1c). In contrast, WM793 and SK-MEL-28 cells were resistant to apoptosis on poly-L-lysine. Consistent with the expression of B-RAF^{V600E} in WM793, levels of phosphoERK1/2 were higher in WM793 compared to NHEM and Sbc12 (Supplementary Figure S1); phosphoERK1/2 levels detected in Sbc12 may reflect autocrine growth factor

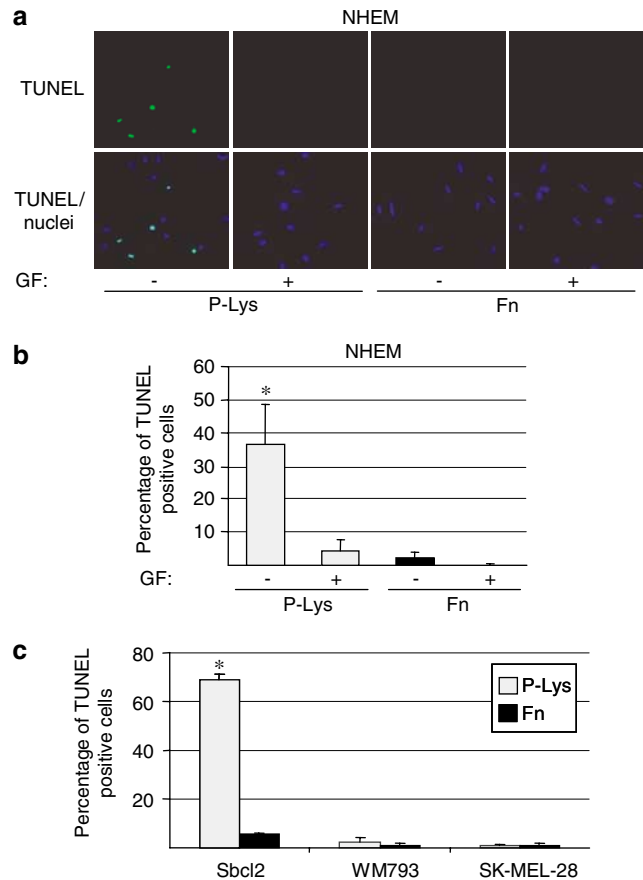


Figure 1 Expression of B-RAF^{V600E} correlates with protection from anoikis. (a) Normal human epidermal melanocytes cells were plated on either poly-L-lysine (P-Lys) or fibronectin (Fn)-coated coverslips in either serum-free (–) or growth factor-supplemented (+) medium. After 48 h, cells were processed for TdT-mediated dUTP nick-end labeling (TUNEL) staining. (b) Averages and standard deviations from three independent experiments. The asterisk indicates statistical significance as determined by a two-tailed unpaired *t*-test ($P < 0.01$). (c) Sbc12, WM793 and SK-MEL-28 cells were plated on P-Lys or Fn for 48 h in the absence of serum/growth factors and then processed for TUNEL staining. The asterisk indicates statistical significance as determined by a two-tailed, unpaired *t*-test comparing Sbc12/P-Lys to Sbc12/Fn, WM793/P-Lys or SK-MEL-28/P-Lys ($P < 0.001$).

production (Davies *et al.*, 2002; Satyamoorthy *et al.*, 2003). These data along with the data from NHEM correlate the expression of mutant B-RAF to resistance to anoikis.

Adhesion and B-RAF–MEK pathways cooperate to protect melanoma cells from anoikis

B-RAF is a potent activator of MEK–ERK1/2 signaling, a pathway that is important for cell survival in many cell types (Khwaja *et al.*, 1997; Le Gall *et al.*, 2000; Jost *et al.*, 2001; Howe *et al.*, 2002); therefore, we sought to determine if MEK activity was important for melanocyte and for melanoma cell survival. NHEM, Sbc12, WM35 and WM793 cells were treated with or without the MEK inhibitor, U0126, and plated on either agar or fibronectin. Replating on agar maintains cells in non-adherent conditions and reduces concerns

regarding melanoma cells producing matrix proteins and spreading on poly-L-lysine. WM35 cells were used in these experiments because they display radial growth phase cell properties similar to that of Sbc12 cells; however, in contrast to Sbc12 cells, they harbor the B-RAF^{V600E} mutation (Davies *et al.*, 2002; Satyamoorthy *et al.*, 2003). Apoptosis was measured by flow cytometry for cleaved caspase 3, activation of which is a key event in the apoptotic cascade.

Consistent with the data from TUNEL assays, NHEM and Sbc12 cells displayed high levels of staining for cleaved caspase 3 when replated on agar (Figure 2, top panels). Treatment of these cells with U0126 did not promote cleavage of caspase 3 on fibronectin or enhance cleavage of caspase 3 in non-adherent conditions. In WM35 and WM793 cells, U0126 did not promote cleavage of caspase 3 on fibronectin but did enhance staining of cleaved caspase 3 in non-adherent conditions (Figure 2, lower panels). These data indicate the existence of two pathways regulating cell survival in melanoma cells: one pathway that is MEK-dependent but adhesion-independent and the other that is adhesion-dependent but MEK-independent.

B-RAF is necessary for melanoma cell resistance to anoikis

B-RAF, an upstream activator of MEK, is frequently mutated in melanoma (Davies *et al.*, 2002). To determine whether B-RAF is required to protect B-RAF^{V600E}-expressing WM793 melanoma cells from anoikis, we utilized RNA interference. Two small-interfering RNAs (siRNAs) targeting different sequences in

B-RAF were used: one to target the region containing the V600E mutation (B-RAF^{V600E}) (Calipel *et al.*, 2003); the other to target a sequence contained within both mutant and wild-type B-RAF (B-RAF^{#1}). Transfection with either B-RAF^{V600E} or B-RAF^{#1} siRNA led to a decrease in B-RAF protein levels as compared to control transfected cells (Figure 3a). Notably, the levels of other RAF isoforms, A-RAF and C-RAF, were unaffected confirming the specificity of the B-RAF knockdown. B-RAF protein levels were not fully eliminated in the B-RAF^{V600E} siRNA-transfected cells, perhaps because these cells are heterozygous for the V600E mutation and the wild-type-B-RAF was less efficiently down-regulated by this siRNA. Concomitant with decreased B-RAF protein expression, levels of phosphorylated MEK1/2 were inhibited upon knockdown of B-RAF.

Next, we measured cleaved caspase 3 levels in control and B-RAF knockdown WM793 cells plated on either agar or fibronectin in the absence of growth factors. Both control and B-RAF knockdown cells plated on fibronectin displayed low levels of staining for cleaved caspase 3 (Figure 3b). On agar, B-RAF but not control knockdown cells showed high-level staining for cleaved caspase 3. Similar effects were observed with both B-RAF-targeting siRNAs, although the B-RAF^{#1} siRNA was more potent at inducing susceptibility to anoikis than the B-RAF^{V600E} siRNA (Figure 3c). Consistent with data from cleaved caspase 3 assays, TUNEL staining was enhanced in B-RAF knockdown cells replated on poly-L-lysine (Figure 3d). Together, these results demonstrate that B-RAF is necessary for protection of WM793 cells from anoikis.

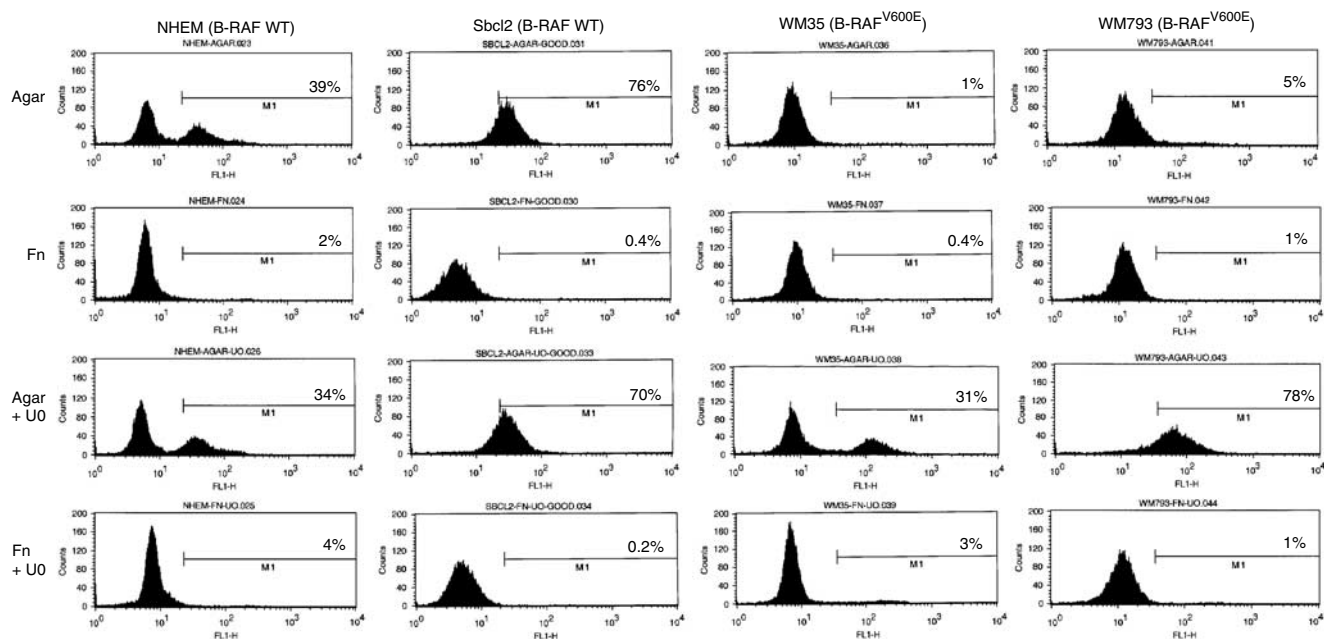


Figure 2 Both adhesion and MEK–ERK1/2 signaling protect melanoma cells from apoptosis. Wild-type B-RAF-expressing (normal human epidermal melanocytes and Sbc12) and mutant B-RAF-expressing (WM35 and WM793) cells were plated on either agar or fibronectin in the absence of growth factors and treated with or without 5 μ M U0126, as indicated, for 48 h. Cleaved caspase 3 levels were determined by flow cytometry. The fluorescence intensity is measured on the x axis and cell counts on the y axis. The percentages of cells staining positive for each condition are indicated. The results are representative of three independent experiments.

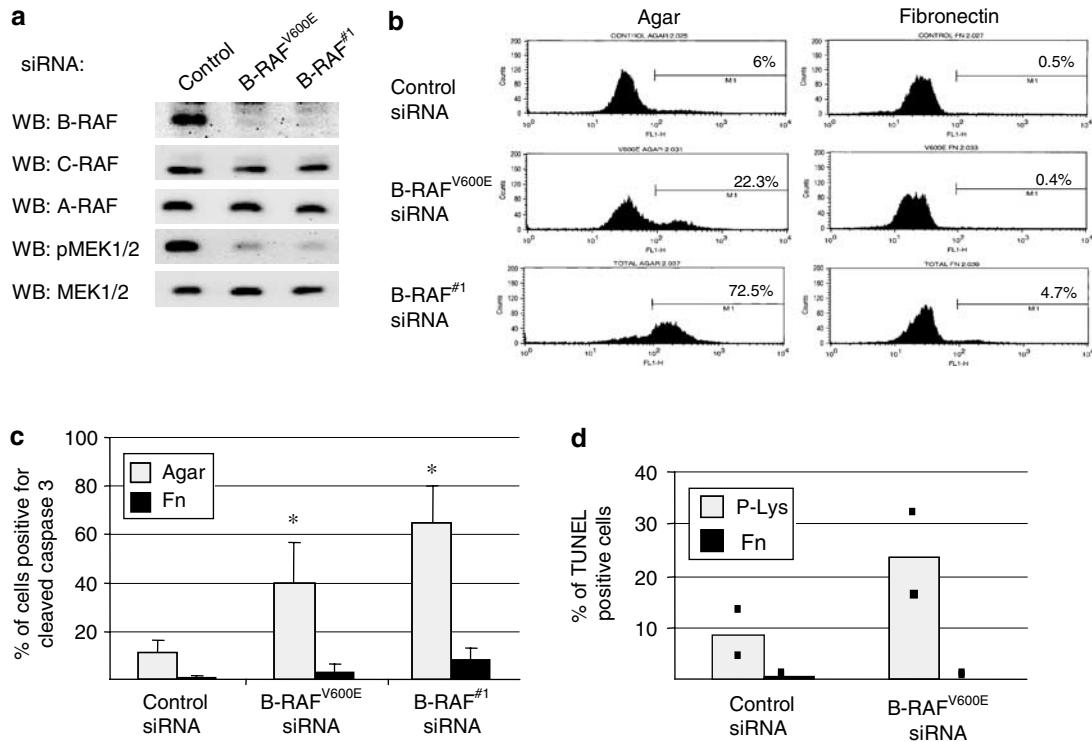


Figure 3 B-RAF is necessary for melanoma cell resistance to anoikis. WM793 cells were transfected with control, B-RAF^{V600E} or B-RAF^{#1} siRNA, as indicated. (a) Seventy-two hours post-transfection, cells were analysed by Western blotting for expression of B-RAF, C-RAF, A-RAF, phospho-MEK1/2 and total MEK1/2. (b) Following transfection, WM793 cells were replated onto either agar- or fibronectin (Fn)-coated dishes for 48 h in serum-free media and analysed for apoptosis by detection of cleaved caspase 3 by flow cytometry. The fluorescence intensity is measured on the x axis and cell counts on the y axis. The percentages of cells staining positive in each condition are indicated. (c) Quantitation of the data from (b) is presented as the average percentage of cells staining for cleaved caspase 3 as determined by comparing fluorescence intensity to the negative control from four independent experiments. The asterisk denotes statistical significance as determined by a two-tailed unpaired *t*-test comparing B-RAF^{V600E}-agar and control-agar ($P < 0.005$) and B-RAF^{#1}-agar and control-agar ($P < 0.001$). (d) Following transfection, WM793 cells were replated onto either P-Lys- or Fn-coated coverslips for 48 h and analysed for apoptosis by TdT-mediated dUTP nick-end labeling (TUNEL) staining and immunofluorescence. Total numbers of cells and those positive for TUNEL staining were counted in five randomly selected fields, with at least 500 cells counted per coverslip. The data presented are the means of two independent experiments. The individual data points are located.

B-RAF knockdown WM793 cells are protected from apoptosis on fibronectin but not collagen

Anoikis can result not only from loss of attachment but also from adhesion to an inappropriate matrix. For example, culture of melanocytes in type I collagen gels induces apoptosis (Alanko *et al.*, 1999; Alanko and Saksela, 2000). During the vertical growth phase, melanoma cells invade into the dermis, a matrix composed primarily of type I collagen but also containing fibronectin deposits. We analysed the role of collagen I on cell survival in WM793 cells. Control knockdown cells displayed low levels of caspase 3 staining on fibronectin and collagen I (Figure 4a). As seen previously, B-RAF knockdown WM793 cells displayed low levels of caspase 3 staining on fibronectin; however, replating on collagen I increased cleavage of caspase 3 in cells transfected with either of the B-RAF targeting siRNAs (Figure 4a and quantitated in Figure 4b). Similar results were observed on 3D collagen gels as seen on 2D collagen (data not shown).

The inability of collagen to promote survival was not due to lack of cell attachment, as WM793 cells attached efficiently to both collagen I and fibronectin and spread on both matrices, as determined by immunofluorescence staining and Western blotting for focal adhesion kinase (FAK) tyrosine 397 phosphorylation (Figure 5a and b). There was, however, a delay of approximately 15 min in the attachment, spreading and FAK phosphorylation in cells plated on collagen I compared to fibronectin. Although the focal adhesions were smaller on collagen I than that on fibronectin, within 30 min the cells had efficiently spread and induced tyrosine phosphorylation of FAK on both matrix substrates. WM793 cells express $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins, the main receptors for collagen I, and B-RAF knockdown did not dramatically alter the levels of these integrins (Figure 5c). Experiments with function-blocking antibodies to $\alpha 1$ and $\alpha 2$ indicated that both integrins contributed to attachment and spreading on collagen (Figure 5d). These data indicate that WM793 adhesion to collagen I does not promote survival signals.

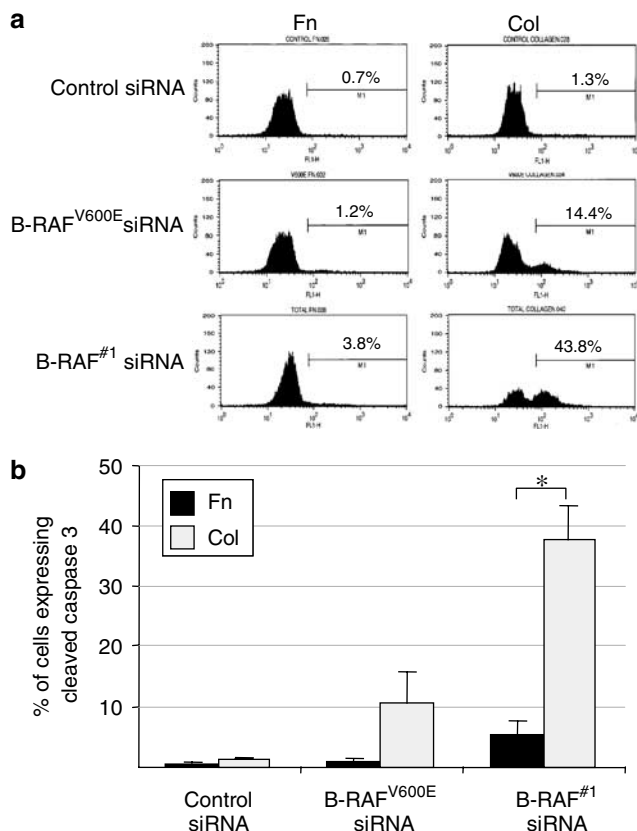


Figure 4 Adhesion to fibronectin (Fn) but not collagen protects B-RAF knockdown WM793 cells from apoptosis. (a) WM793 cells were transfected with control, B-RAF^{V600E} or B-RAF^{#1} siRNA. After 72 h, cells were replated on Fn or type I collagen (Col)-coated dishes for 48 h in serum-free medium. The percentages of cells staining positive for cleaved caspase 3 as determined by flow cytometry, are indicated. The fluorescence intensity is measured on the x axis and cell counts on the y axis. (b) Quantitation of three independent experiments. The increased percentage of cleaved caspase 3-positive cells seen in both B-RAF^{V600E} and B-RAF^{#1} siRNA-transfected WM793 cells on Col as compared to Fn is statistically significant (two-tailed unpaired *t*-test, $P < 0.001$).

AKT is activated by adhesion to fibronectin but not collagen I in WM793 cells

In order to identify the signaling pathway that mediates survival of B-RAF knockdown cells on fibronectin, we analysed AKT signaling. Control and B-RAF knockdown WM793 cells were plated onto poly-L-lysine, fibronectin or collagen I for 1 h and cell lysates were analysed by Western blotting for levels of phosphorylated AKT at the two activation sites, threonine 308 and serine 473. Notably, AKT was phosphorylated only when the cells were plated onto fibronectin but not poly-L-lysine or collagen I, and this phosphorylation was independent of B-RAF expression (Figure 6a). Similar results have been observed in SK-MEL-28 cells (Spofford *et al.*, manuscript submitted), showing that fibronectin-mediated activation of AKT is not limited to one melanoma cell line.

Use of an antibody to phosphorylated AKT motifs (R/K-X-R/K-X-X-S/T) recognized a doublet of approximately 44/46 kDa in lysates from cells plated on

fibronectin but not poly-L-lysine or collagen (Figure 6b). GSK3 β is a protein phosphorylated and inactivated by AKT (Cross *et al.*, 1995), is approximately 46 kDa and has been shown to play a role in apoptosis (Pap and Cooper, 1998). Using a phosphospecific GSK3 antibody, we found that GSK3 β was phosphorylated only in cells plated onto fibronectin. To determine if GSK3 is an important regulator of anoikis in these cells, we utilized siRNA to knock down GSK3. Although we obtained an 80% decrease in GSK3 β protein levels, we did not observe protection from anoikis in cells co-transfected with B-RAF and GSK3 siRNAs (Supplementary Figure S2), arguing against a role of GSK3 β in mediating apoptosis downstream of AKT in melanoma cells. In sum, these results indicate that fibronectin-mediated activation of AKT promotes survival signaling in melanoma cells.

PI-3 kinase signaling is necessary for adhesion-mediated survival in B-RAF knockdown WM793 cells

AKT activation is regulated by PI-3 kinase signaling. To determine if PI-3 kinase activity is necessary for the survival of WM793 cells attached to fibronectin, we used the PI-3 kinase inhibitor, LY294002. B-RAF knockdown WM793 cells were treated with 10 μ M LY294002, which is sufficient to reduce AKT activation (Figure 7a), and plated onto either fibronectin-coated dishes or agar. As observed previously, fibronectin was sufficient to protect B-RAF knockdown cells (Figure 7b). However, the combination of B-RAF knockdown and LY294002 treatment enhanced cleaved caspase 3 staining on fibronectin (Figure 7b and c). The percentage of B-RAF knockdown cells staining positive for cleaved caspase 3 was less after LY294002 than in non-adherent conditions. These data suggest that fibronectin-mediated PI-3 kinase signaling provides a second survival cue that, in the absence of B-RAF signaling, protects melanoma cells from apoptosis.

Discussion

In this study, we showed that two survival pathways operate in melanoma cells to provide protection from anoikis: B-RAF–MEK and PI-3 kinase signaling. B-RAF was mutated in approximately 70% of melanomas leading to constitutive MEK–ERK1/2 signaling (Davies *et al.*, 2002; Conner *et al.*, 2003; Satyamoorthy *et al.*, 2003) and PI-3 kinase–AKT signaling was frequently deregulated through either mutations in PTEN or overexpression of AKT3 (Stahl *et al.*, 2003, 2004).

A particularly interesting finding from our studies is that activation of AKT was substrate-dependent in melanoma cells. WM793 cells displayed decreased expression of PTEN and enhanced AKT activity compared to melanocytes (Spofford, *et al.*, manuscript submitted); hence, fibronectin regulation of this pathway is permissive for PI-3 kinase signaling to AKT. A role for PI-3 kinase–AKT signaling in adhesion-dependent survival is consistent with earlier studies in

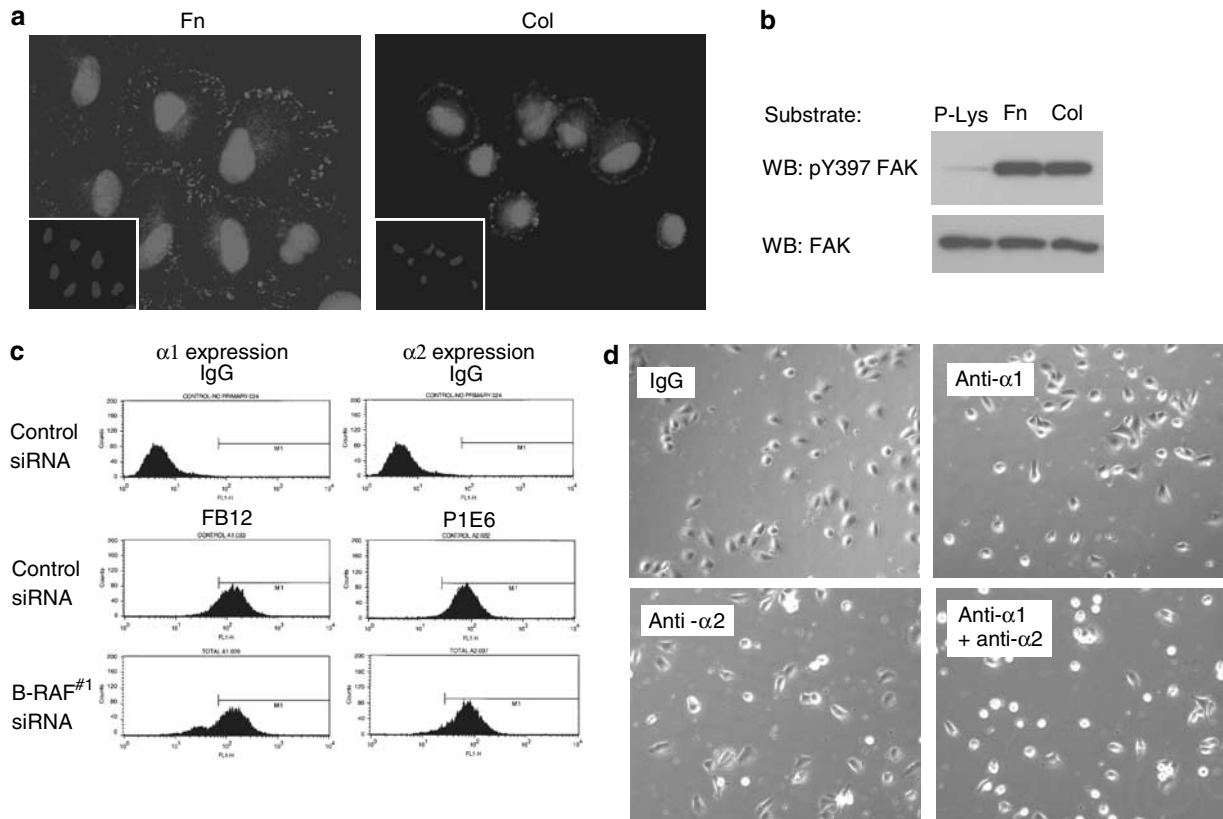


Figure 5 WM793 cells adhere to and spread on both fibronectin (Fn) and collagen (Col). (a) WM793 cells were serum-starved for 16 h and replated onto coverslips pre-coated with either Fn or Col for 30 min. Subsequently, the cells were fixed, permeabilized and stained for phospho-Y397 FAK. Staining was detected with Fluor488-conjugated secondary antibodies and immunofluorescence. Hoechst reagent was used to detect nuclear staining (insets). (b) Lysates from WM793 cells replated onto either P-Lys-, Fn- or Col-coated dishes for 30 min were analysed by Western blot for FAK and phospho-Y397 FAK. (c) WM793 cells were transfected with either control or B-RAF^{#1} siRNA and after 72 h, the cells were serum-starved for 16 h and harvested for detection of $\alpha 1$ or $\alpha 2$ integrin subunits by flow cytometry. The fluorescence intensity is measured on the *x* axis and the cell counts on the *y* axis. (d) WM793 cells were serum-starved for 16 h followed by incubation with the indicated antibody for 1 h and replated onto collagen-coated dishes. Images were taken 1 h after replating.

epithelial cells (Khwaja *et al.*, 1997; Marshman *et al.*, 2003). AKT3 likely regulates these effects as it has been demonstrated to play a role in melanoma survival (Stahl *et al.*, 2004). Importantly in our work, AKT activation was independent of B-RAF expression. Fibronectin regulation of AKT activation may be particularly relevant at the transition from radial to vertical phase growth that is associated with survival and growth in the dermis. Interestingly, type I collagen, the major dermal matrix component, was unable to promote survival in the absence of B-RAF expression. These results highlight potential differences in integrin heterodimer signaling (Giancotti and Ruoslahti, 1999). Consistent with fibronectin providing a protective signal, fibronectin mRNA expression is enhanced in some melanoma cell lines via induction of the transcription factor Egr-1 (Gaggioli *et al.*, 2005).

We showed that either matrix adhesion or exogenous growth factors are sufficient to protect human melanocytes from apoptosis. These data are consistent with previous findings demonstrating that melanocytes are protected from apoptosis when plated on fibronectin,

laminin or collagen III (Scott *et al.*, 1997) but not type I collagen gels (Alanko *et al.*, 1999; Alanko and Saksela, 2000). In the absence of adhesion and subsequent activation of AKT, our data demonstrated that B-RAF^{V600E} signaling is sufficient for survival of melanoma cells. These findings extend the work of Scott *et al.*, 1997, who used the B-RAF^{V600E}-expressing metastatic melanoma SK-MEL-28 cell line, by using a panel of cell lines from different stages of melanoma progression and showing a requirement for B-RAF expression and MEK activity for survival in non-adherent conditions. Previous studies yielded differing results with respect to the requirement for B-RAF for melanoma cell survival (Hingorani *et al.*, 2003; Karasarides *et al.*, 2004; Sumimoto *et al.*, 2004). Our data showed that knock-down of B-RAF has only a minor effect on inducing apoptosis in cells adherent to fibronectin.

The majority of RAF studies have focused on C-RAF and shown that it provides protection from anoikis in epithelial and fibroblast cell lines (Le Gall *et al.*, 2000; Schulze *et al.*, 2001). C-RAF mediates protection from apoptosis, either via an MEK-ERK1/2-independent

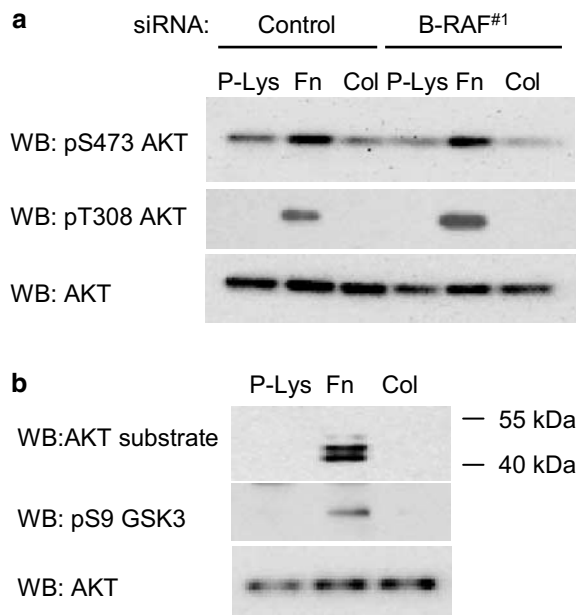


Figure 6 Adhesion to fibronectin (Fn) but not collagen (Col) enhances AKT phosphorylation and activity in WM793 cells. **(a)** WM793 cells were transfected with control, B-RAF^{V600E} or B-RAF^{#1} siRNA. After 72 h, serum-starved cells were replated onto poly-L-lysine, Fn or Col for 1 h in the absence of serum. The cells were lysed and analysed for phosphorylation of AKT at both Thr 308 and Ser 473 and for total AKT levels by Western blot. **(b)** WM793 cells were processed, as above. Cell lysates were analysed for phosphorylated AKT substrates, using an antibody that detects proteins containing the phosphorylated (R/K)X(R/K)XX(S/T) motif and phospho (Ser9) GSK3 β . Total AKT levels are shown as a loading control.

adaptor function (Huser *et al.*, 2001; O'Neill *et al.*, 2004), highlighting possible differences between the modes of C-RAF and B-RAF protection, or through MEK–ERK1/2 signaling (Schulze *et al.*, 2004). C-RAF is rarely mutated in cancer; nevertheless, it may play an important role in a subset of melanomas expressing B-RAF mutants that are not activating but rather bind to and promote C-RAF signaling (Wan *et al.*, 2004).

Future studies will determine whether B-RAF–MEK and PI-3 kinase act independently or cooperate to control an individual apoptotic regulator. Putative targets of such cooperative regulation include the proapoptotic Bcl2 family proteins, Bcl2 interacting mediator of cell death (Bim) and Bad that can be phosphorylated at different sites by ERK1/2 and AKT signaling (Datta *et al.*, 2000; Biswas and Greene, 2002; Ley *et al.*, 2003; She *et al.*, 2005; Qi *et al.*, 2006). Phosphorylation of these proteins targets them for proteasomal degradation and/or 14-3-3-mediated sequestration (Datta *et al.*, 2000; Ley *et al.*, 2003; She *et al.*, 2005; Qi *et al.*, 2006). Additionally, expression of the longest Bim isoform, Bim EL, is known to be downregulated by the ERK1/2 pathway at the level of transcription in an adhesion-dependent manner in breast epithelial cells (Reginato *et al.*, 2003). Studies from mouse models implicate Bim in melanocyte cell death as the loss of coat color in Bcl2-deficient mice

is reversed in Bcl2/Bim double knockouts (Bouillet *et al.*, 2001). Bad has previously been shown to be regulated by ERK1/2 via RSK-mediated phosphorylation at serine 112 in melanoma cells. Notably, a Bad S112A mutant renders melanoma cells sensitive to apoptosis (Eisenmann *et al.*, 2003). Alternatively, B-RAF^{V600E} and AKT may function downstream of Bcl-2 family proteins and cytochrome *c* release possibly through control of inhibitor of apoptosis proteins (IAP), a family of proteins that inhibit caspase activation (Erhardt *et al.*, 1999).

In summary, this work illustrates the importance of both PI-3 kinase–AKT and B-RAF–MEK signaling in the survival of melanoma cells and suggests that targeting both pathways may be necessary in therapeutic strategies for later stage melanomas. Future work will determine how fibronectin and collagen signaling differ in melanoma cells and will examine possible coordinate regulation of Bcl-2 and IAP family members by the PI-3 kinase and B-RAF pathways.

Materials and methods

Isolation of normal human epidermal melanocytes and melanoma cell culture conditions

Normal human epidermal melanocytes were isolated from neonatal foreskins according to Albany Medical College Institutional Review Board procedures and maintained as previously described (Conner *et al.*, 2003). Human Sbc12, WM35 and WM793 cells were cultured in MCDB 153 containing 20% Leibovitz L-15 medium, 2% fetal bovine serum and 5 μ g/ml insulin.

Antibodies and inhibitors

The following antibodies were purchased from Cell Signaling Technology, Beverly, MA: anti-phospho-AKT (Thr 308, #4056 and Ser 473, #9271), anti-phospho-(Ser/Thr) AKT substrate (#9611), anti-cleaved caspase 3 (#9661), anti-phospho-ERK (Thr202/Tyr204, #4377) and anti-phospho-MEK (Ser217/221, #9121). Anti-ERK1/2 (sc1647), anti-C-RAF (sc133), and anti-A-RAF (sc407) were purchased from Santa Cruz Biotech, Santa Cruz, CA. B-RAF (612374) and AKT1 (P67220) antibodies were purchased from BD Biosciences. Anti-integrin α 1 (FB12) and anti-integrin α 2 (P1E6) were purchased from Chemicon, Temecula, CA. The phospho-GSK3 β (Ser9) antibody was a kind gift from Andrés Melendez, Albany Medical College. The MEK inhibitor U0126 and the PI-3 kinase inhibitor LY294002 were purchased from Cell Signaling Technology.

siRNA transfections

WM793 cells were transfected with siRNAs using Oligofectamine (Invitrogen, Carlsbad, MA). The following siRNAs (synthesized by Dharmacon, Lafayette, CO) were utilized at 25 nM: non-targeting control, B-RAF^{V600E} (Calipel *et al.*, 2003) and B-RAF^{#1}. Seventy-two hours post-transfection, cells were serum-starved for a further 16 h and then replated for cell adhesion assays.

Cell adhesion assays

Cells were replated onto dishes coated with poly-L-lysine (10 μ g/ml), bactoagar (2%), fibronectin (20 μ g/ml; BD Biosciences) or rat tail type I collagen (20 μ g/ml; BD Biosciences)

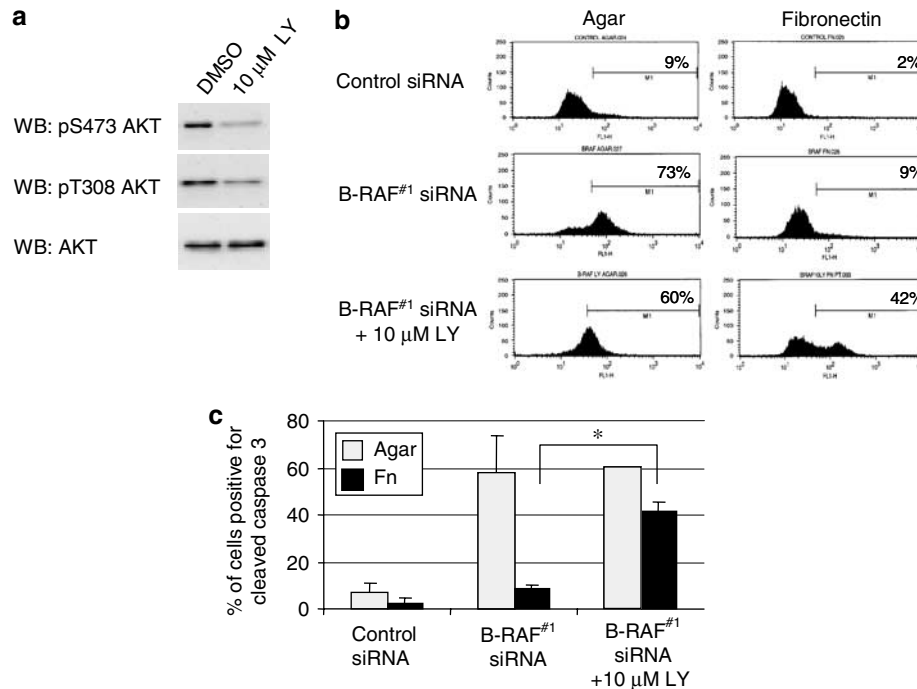


Figure 7 PI-3 kinase–AKT signaling is required for adhesion-mediated survival in WM793 cells. **(a)** WM793 cells were serum-starved and treated with either DMSO or 10 μ M LY294002 for 96 h. The lysates were analysed by Western blot for phospho-AKT (Thr308 and Ser473) and total AKT as a loading control. **(b)** WM793 cells were transfected with either control or B-RAF^{#1} siRNA. After 72 h, the cells were serum-starved and treated with either 10 μ M LY294002 or DMSO (vehicle control). Following 16 h, cells were replated onto either agar or fibronectin (Fn) for 48 h in serum-free medium with or without 10 μ M LY294002. The cells were harvested and analysed for cleaved caspase 3 by flow cytometry. The fluorescence intensity is measured on the x axis and cell counts on the y axis. The percentages of cells staining positive for cleaved caspase 3 are indicated. **(c)** Quantitation of three independent experiments. The asterisk denotes statistical significance as determined by a two-tailed unpaired *t*-test comparing B-RAF^{#1}-transfected cells on Fn and treated with or without 10 μ M LY294002 ($P < 0.001$).

for 48 h in serum-free MCDB 153 medium containing 0.5% bovine serum albumin (BSA). Cells were then processed for Western blot analysis or apoptosis assays. For antibody blocking experiments, cells were incubated with 5 μ g/ml anti-integrin antibody or murine control IgG at 37°C for 1 h. Treated cells were then replated onto dishes coated with rat tail type I collagen for 1 h followed by visualization by phase microscopy.

Western blotting

Cells were washed and lysed directly in Laemmli sample buffer. Lysates were separated on SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Proteins were detected using the indicated primary antibody and the signal was detected using peroxidase-conjugated secondary antibody followed by development using chemiluminescence substrate (Pierce, Rockford, IL). Chemiluminescence was detected using a Fluor-S Multi-Imager (BioRad, Hercules, CA) and was quantitated using Quantity-One software.

Apoptosis assays

TdT-mediated UTP nick end labeling staining was carried out using the DeadEnd Fluorometric TUNEL system from Promega, according to the manufacturer's instructions. Total cells and those positive for TUNEL staining were counted in five randomly selected fields, with at least 500 cells counted per coverslip. For analysis of cleaved caspase 3, trypsinized cells

were fixed in 4% methanol-free formaldehyde and permeabilized in methanol overnight. Cells were then washed in PBS/0.5% BSA, blocked in the same buffer and incubated with anti-cleaved caspase 3 antibody for 1 h at 4°C. Washed cells were then incubated with Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes Inc., Eugene, OR) for 30 min at 4°C. Finally, cells were washed and resuspended for flow cytometry analysis on the FACSCalibur (Becton Dickinson Biosciences, San Jose, CA). Data were analysed using Cell Quest (Becton Dickinson). Cells were gated on forward and side scatter to differentiate the cells from debris and 1×10^4 cells were counted for each condition. Data were plotted on a histogram as mean fluorescent intensity versus cell number.

Integrin staining

WM793 cells were washed in PBS/0.5% BSA, blocked in the same buffer and incubated with anti-integrin $\alpha 1$ (FB12) antibody, anti-integrin $\alpha 2$ (P1E6) antibody or murine control IgG for 1 h at 4°C. Washed cells were then incubated with Alexa Fluor 488-conjugated goat anti-mouse antibody (Molecular Probes) for 30 min at 4°C. Finally, cells were washed and resuspended for flow cytometry analysis on the FACS Calibur (Becton Dickinson).

Statistical analysis

Statistical analyses were performed with a two-tailed unpaired *t*-test. *P*-values < 0.01 were considered statistically significant.

Abbreviations

Bad, Bcl-X_L/Bcl-2-associated death promoter; Bim, Bcl-2 interacting mediator of cell death; FAK, focal adhesion kinase; IAP, inhibitor of apoptosis protein; NHEM, normal human epidermal melanocyte; PI-3 kinase, phosphoinositide-3 kinase; PTEN, phosphatase and tensin homolog; siRNA, small interfering RNA; TUNEL, TdT-mediated dUTP nick-end labeling.

References

- Alanko T, Rosenberg M, Saksela O. (1999). *J Invest Dermatol* **113**: 111–116.
- Alanko T, Saksela O. (2000). *J Invest Dermatol* **115**: 286–291.
- Alonso SR, Ortiz P, Pollan M, Perez-Gomez B, Sanchez L, Acuna MJ *et al.* (2004). *Am J Pathol* **164**: 193–203.
- Bataille V. (2003). *Eur J Cancer* **39**: 1341–1347.
- Bhatt KV, Spofford LS, Aram G, McMullen M, Pumiglia K, Aplin AE. (2005). *Oncogene* **12**: 3459–3471.
- Biswas SCA, Greene LA. (2002). *J Biol Chem* **277**: 49511–49516.
- Bouillet P, Cory S, Zhang LC, Strasser A, Adams JM. (2001). *Dev Cell* **1**: 645–653.
- Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS *et al.* (1999). *Cell* **96**: 857–868.
- Calipel A, Lefevre G, Pouponnot C, Mouriaux F, Eychene A, Mascarelli F. (2003). *J Biol Chem* **278**: 42409–42418.
- Conner SR, Scott G, Aplin AE. (2003). *J Biol Chem* **278**: 34548–34554.
- Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. (1995). *Nature* **378**: 785–789.
- Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y *et al.* (1997). *Cell* **91**: 231–241.
- Datta SR, Katsov A, Hu L, Petros A, Fesik SW, Yaffe MB *et al.* (2000). *Mol Cell* **6**: 41–51.
- Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S *et al.* (2002). *Nature* **417**: 949–954.
- Davies MA, Lu Y, Sano T, Fang X, Tang P, LaPushin R *et al.* (1998). *Cancer Res* **58**: 5285–5290.
- del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. (1997). *Science* **278**: 687–689.
- Dong J, Phelps RG, Qiao R, Yao S, Benard O, Ronai Z *et al.* (2003). *Cancer Res* **63**: 3883–3885.
- Eisenmann KM, VanBrocklin MW, Staffend NA, Kitchen SM, Koo H-M. (2003). *Cancer Res* **63**: 8330–8337.
- Erhardt P, Schremser EJ, Cooper GM. (1999). *Mol Cell Biol* **19**: 5308–5315.
- Frisch SM, Francis H. (1994). *J Cell Biol* **124**: 619–626.
- Fukazawa H, Noguchi K, Murakami Y, Uehara Y. (2002). *Mol Cancer Ther* **1**: 303–309.
- Gaggioli C, Deckert M, Robert G, Abbe P, Batoz M, Ehrengreber MU *et al.* (2005). *Oncogene* **24**: 1423–1433.
- Giancotti FG, Ruoslahti E. (1999). *Science* **285**: 1028–1032.
- Gill M, Celebi JT. (2005). *J Am Acad Dermatol* **53**: 108–114.
- Guldberg P, thor Straten P, Birck A, Ahrenkiel V, Kirkin AF, Zeuthen J. (1997). *Cancer Res* **57**: 3660–3663.
- Hingorani SR, Jacobetz MA, Robertson GP, Herlyn M, Tuveson DA. (2003). *Cancer Res* **63**: 5198–5202.
- Howe AK, Aplin AE, Juliano RL. (2002). *Curr Opin Genet Dev* **12**: 30–35.
- Huser M, Luckett J, Chiloeches A, Mercer K, Iwobi M, Giblett S *et al.* (2001). *EMBO J* **20**: 1940–1951.

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- Jost M, Huggett TM, Kari C, Rodeck U. (2001). *Mol Biol Cell* **12**: 1519–1527.
- Kane LP, Shapiro VS, Stokoe D, Weiss A. (1999). *Curr Biol* **9**: 601–604.
- Karasarides M, Chiloeches A, Hayward R, Niculescu-Duvaz D, Scanlon I, Friedlos F *et al.* (2004). *Oncogene* **23**: 6292–6298.
- Khwaja A, Rodriguez-Viciano P, Wennstrom S, Warne PH, Downward J. (1997). *EMBO J* **16**: 2783–2793.
- Koh HK. (1991). *N Engl J Med* **325**: 171–182.
- Le Gall M, Chambard JC, Breittmayer JP, Grall D, Pouyssegur J, Van Obberghen-Schilling E. (2000). *Mol Biol Cell* **11**: 1103–1112.
- Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ. (2003). *J Biol Chem* **278**: 18811–18816.
- Marshman E, Green KA, Flint DJ, White A, Streuli CH, Westwood M. (2003). *J Cell Sci* **116**: 675–682.
- Mayo LD, Donner DB. (2001). *Proc Natl Acad Sci USA* **98**: 11598–11603.
- O'Neill E, Rushworth L, Baccharini M, Kolch W. (2004). *Science* **306**: 2267–2270.
- Pap M, Cooper GM. (1998). *J Biol Chem* **273**: 19929–19932.
- Pollock PM, Harper UL, Hansen KS, Yudt LM, Stark M, Robbins CM *et al.* (2003). *Nat Genet* **33**: 19–20.
- Qi X-J, Wildey GM, Howe PH. (2006). *J Biol Chem* **281**: 813–823.
- Reginato MJ, Mills KR, Paulus JK, Lynch DK, Sgroi DC, Debnath J *et al.* (2003). *Nat Cell Biol* **6**: 6.
- Satyamoorthy K, Li G, Gerrero MR, Brose MS, Volpe P, Weber BL *et al.* (2003). *Cancer Res* **63**: 756–759.
- Schulze A, Lehmann K, Jefferies HBJ, McMahon M, Downward J. (2001). *Genes Dev* **15**: 981–994.
- Schulze A, Nicke B, Warne PH, Tomlinson S, Downward J. (2004). *Mol Biol Cell* **15**: 3450–3463.
- Scott G, Cassidy L, Busacco A. (1997). *J Invest Dermatol* **108**: 147–153.
- She QB, Solit DB, Ye Q, O'Reilly KE, Lobo J, Rosen N. (2005). *Cancer Cell* **8**: 287–297.
- Stahl JM, Cheung M, Sharma A, Trivedi NR, Shanmugam S, Robertson GP. (2003). *Cancer Res* **63**: 2881–2890.
- Stahl JM, Sharma A, Cheung M, Zimmerman M, Cheng JQ, Bosenberg MW *et al.* (2004). *Cancer Res* **64**: 7002–7010.
- Sumimoto H, Miyagishi M, Miyoshi H, Yamagata S, Shimizu A, Taira K *et al.* (2004). *Oncogene* **23**: 6031–6039.
- Tsao H, Zhang X, Benoit E, Haluska FG. (1998). *Oncogene* **16**: 3397–3402.
- Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM *et al.* (2004). *Cell* **116**: 855–867.
- Wang JM, Chao JR, Chen W, Kuo ML, Yen JJ, Yang-Yen HF. (1999). *Mol Cell Biol* **19**: 6195–6206.
- Wellbrock C, Ogilvie L, Hedley D, Karasarides M, Martin J, Niculescu-Duvaz D *et al.* (2004). *Cancer Res* **64**: 2338–2342.

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