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p66^{Shc} restrains Ras hyperactivation and suppresses metastatic behavior

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Abstract

Normal tissue cells survive and proliferate only while anchored to solid substrate. Conversely, transformed cells both survive and proliferate following detachment, having lost attachment context through unclear mechanisms. p66^{Shc} is a focal adhesion-associated protein that reports cell attachment through a RhoA-dependent mechanosensory test. We find that human small cell lung cancer (SCLC) cells and mouse Lewis lung carcinoma (LLC), which display aggressive metastatic behavior, lack both p66^{Shc} and retinoblastoma (pRB) and bypass anoikis. Re-expression of p66^{Shc} in these cells restores anoikis and provides striking protection from metastasis by LLC cells *in vivo*. Notably, knockdown of p66^{Shc} in normal epithelial cells leads to unrestrained Ras activation, preventing anoikis through downstream suppression of RhoA but blocking proliferation in a pRB-dependent manner, thus mimicking oncogenic Ras. Conversely, LLC and SCLC cells display constitutive Ras activation necessary to bypass anoikis, which is reversed by re-expression of p66^{Shc}. p66^{Shc} therefore coordinates Ras-dependent control of proliferation and anchorage sensation, which can be defeated in the evolution of highly metastatic tumors by combined loss of both p66^{Shc} and pRB.

Keywords

anoikis; Shc; pRB; metastasis; small cell lung cancer

Introduction

Differentiated epithelial and endothelial cells have strong attachment requirements, presumably to prevent cellular vagrancy and consequent tissue disorganization. During transformation, cells lose such anchorage context and not only survive but also proliferate without attachment to solid matrix. Certain pathways downstream from integrins may influence detachment signals. Ectopic expression of integrin-linked kinase, TrkB, or oncogenic forms of Src and Ras have been shown to abrogate anoikis (Frisch and Francis, 1994; Frisch *et al.*, 1996; Attwell *et al.*, 2000; McFall *et al.*, 2001; Douma *et al.*, 2004). In addition, during certain stages of development and tissue repair, normal cells also bypass

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Conflict of interest
The authors declare no conflict of interest.

anoikis in order to migrate, suggesting native regulation of endogenous proteins that control attachment sensation.

The adapter protein p66^{Shc} was recently found to localize to focal adhesions and permit attachment sensation by imposing RhoA-dependent tension across these integrin anchorage points, creating a mechanosensory test of anchorage (Ma *et al.*, 2007). Upon detachment of cells from their underlying matrix, this p66-dependent tension test is met with load failure, initiating apoptosis. In contrast, the absence of p66^{Shc} allows both survival and proliferation of unanchored 293 cells. In this study, we test the relevance of p66^{Shc} to metastatic capacity and control of proliferation in both normal and transformed cells. Surprisingly, we find that p66^{Shc} functions to restrain Ras and allow its physiologic regulation. Upon loss of p66^{Shc} expression, endogenous Ras becomes hyperactivated and phenocopies oncogenic Ras, suppressing proliferation through retinoblastoma (pRB) in normal cells but allowing anchorage independence *in vitro* and aggressive metastasis of lung cancer cells *in vivo*.

Results

p66^{Shc} acts upstream of RhoA to control anoikis in normal and lung cancer cells

The *SHC* gene encodes three proteins that differ in the length of the amino terminus (p46, p52 and p66). We examined Shc proteins in three highly metastatic cancer cells, the mouse Lewis lung carcinoma (LLC) and two lines of human small cell lung cancer (SCLC; H69 and H209) harvested from metastatic sites. In contrast to primary normal human bronchial epithelial (NHBE) cells, which express all three forms of Shc, the LLC, H69 and H209 cell lines lacked detectable p66^{Shc} (Figure 1a). Re-expression of p66^{Shc} through lentiviral transduction caused constitutive activation of RhoA in all three cell lines (Figure 1b), consistent with the ability of p66^{Shc} to act upstream of RhoA in attachment sensing (Ma *et al.*, 2007). Indeed, re-expression of p66^{Shc} restored anoikis in floating cultures of all three cancer cell lines, which was reversed by RhoA(N19) (Figure 1c). Conversely, primary epithelial and endothelial cells (NHBE and human umbilical vein endothelial cells) express p66^{Shc} and display basal activation of RhoA. Knockdown of p66^{Shc} by lentiviral delivery of short hairpin RNA (shRNA) completely suppressed basal RhoA activity (Figure 1d). NHBE cells, typical of normal epithelial cells, have intact anoikis mechanisms with significant cell death in floating culture for 16 h (Figure 1e). Knockdown of p66^{Shc} or expression of RhoA(N19) significantly blocked detachment-induced cell death (Figure 1e). A second small hairpin against p66^{Shc} (shRNA(2)) confirmed that p66^{Shc} is necessary for anoikis (Figure 1f). Thus, in both normal and malignant epithelium, p66^{Shc} acts upstream of RhoA to signal attachment status and initiate anoikis following detachment.

p66^{Shc} prevents lung metastasis *in vivo*

To test the significance of p66^{Shc}-induced anoikis for metastatic capacity *in vivo*, we stably transfected LLC cells with either native p66^{Shc} or p66(S36E), using retroviral transduction with the PINCO plasmid that expresses green fluorescent protein (GFP; Grignani *et al.*, 1998). The S36E mutation lies within the N-terminal CH2 domain unique to the p66^{Shc} isoform, and abrogates its attachment-sensing function (Ma *et al.*, 2007). As expected, LLC cells expressing p66^{Shc} had increased cell death following detachment but not while attached, whereas p66(S36E) had little effect (Figure 2a). Expression of p66^{Shc} or p66(S36E) had no significant effect on the proliferation of adherent LLC cells (Figure 2b), suggesting a specific effect on anoikis in these cells.

When injected into the tail vein of C57BL/6 mice, control LLC cells caused lethality in 18/19 mice by 45 days, with a median survival of 25 days (Figure 2c). In all cases, death was accompanied by a heavy lung metastatic burden, easily identified by GFP-positive

masses and verified histologically and by lung weights (Figures 2d–f). In contrast, mice injected with LLC cells expressing $p66^{Shc}$ had minimal mortality, with only 1/21 mice dying from lung metastases. In this group, two mice were killed because of late outgrowths of tumors at the injection site; neither of these mice had detectable metastases in the lungs or abdominal compartment. The single mouse injected with $p66^{Shc}$-expressing LLC cells that died from lung metastases was examined further by dissection of the metastatic nodules. Remarkably, the nodules had completely lost expression of $p66^{Shc}$ despite being GFP positive (Figure 2g). Because these LLC cells were not clonally selected, it is likely that the metastases in this animal represented a subclonal outgrowth of LLC cells, selected for their loss of $p66^{Shc}$. In further support of the specific effect of $p66^{Shc}$ on metastatic capability, the mortality of mice injected with LLC cells expressing p66(S36E) resembled that of mice injected with control LLC cells, with 15/19 animals dying of lung metastases in 45 days for a median survival of 27 days. A representative sampling of metastatic nodules from this group showed no loss of presumably mutant p66 (Figure 1g). Thus, loss of metastatic capacity *in vivo* correlated accurately with anoikis *in vitro* and with expression of $p66^{Shc}$ with an intact CH2 domain.

Absence of $p66^{Shc}$ arrests proliferation through activation of pRB

Because matrix attachment also permits proliferative signaling, we next asked whether loss of $p66^{Shc}$ also influences cell growth. Despite their continued attachment to culture dishes, knockdown of $p66^{Shc}$ effectively blocked proliferation of NHBE cells, suggesting that loss of attachment sensation may cause a default cell cycle arrest (Figure 3a). In contrast, LLC and SCLC cells proliferate despite the absence of $p66^{Shc}$. Notably, ~90% of SCLC tumors are known to lack functional pRB (Wistuba *et al.*, 2001), allowing for the possibility that pRB may normally enforce a proliferative arrest following the loss of $p66^{Shc}$, an effect lost without functional pRB. In support of this hypothesis, expression of the pocket-binding viral protein E7 by NHBE effectively reversed the growth arrest induced by $p66^{Shc}$ knockdown, whereas the nonfunctional E7(Δ21–24) had no effect (Figure 3a). In parallel, $p66^{Shc}$ knockdown caused hypophosphorylation (activation) of endogenous pRB (Figure 3b). The effect of $p66^{Shc}$ knockdown on pRB phosphorylation and proliferation was confirmed with the second shRNA against $p66^{Shc}$ (Figures 3b and c). We further found that LLC, H69 and H209 cells lacked significant pRB expression (Figure 3d). Although expression of $p66^{Shc}$ alone did not reduce proliferation in any of these tumor cell lines, pRB expression reduced proliferation to a variable degree (Figures 3e–g). Strikingly, expression of $p66^{Shc}$ reversed the growth-suppressive effect of pRB in all three cell lines. These data suggest that in both normal and transformed epithelium, $p66^{Shc}$ acts upstream of pRB to suppress its function, allowing cell cycle progression. Thus, in the absence of $p66^{Shc}$, pRB defaults to its hypophosphorylated state to halt proliferation. In SCLC and LLC cells, the absence of $p66^{Shc}$ is tolerated because of the additional loss of pRB, which permits cell growth.

$p66^{Shc}$ restrains K-Ras hyperactivation upstream from pRB

The effect of $p66^{Shc}$ on proliferation appears to be distinct from its effect on anoikis, which is RhoA dependent but pRB independent, as $p66^{Shc}$ restores anoikis in SCLC and LLC cells without pRB (Figure 1c). Because Ras potentially influences both pRB and RhoA, we next asked if Ras acts as a coordination point for both pathways. Notably, SCLC and LLC cells displayed high basal levels of Ras activation (Figure 4a). In all three cell lines, Ras activation was quantitatively decreased by expression of $p66^{Shc}$, indicating suppression of Ras activity by $p66^{Shc}$ independent of pRB. Specifically, $p66^{Shc}$ suppressed K-Ras with no effect on N-Ras. In these cells, H-Ras was not expressed in significant levels (Figure 4b). Conversely, in primary NHBE and human umbilical vein endothelial cells, basal Ras activity was low but became hyperactivated by $p66^{Shc}$ knockdown (Figure 4c). In NHBE cells, this effect was also specific for K-Ras and not H-Ras or N-Ras (Figure 4d). Again,

striking hyperactivation of K-Ras in NHBE cells was replicated using a second shRNA against $p66^{Shc}$ (Figure 4e). Thus, in both normal and malignant cells, $p66^{Shc}$ restrains hyperactivation of K-Ras.

We next asked if the hyperactivation of K-Ras due to loss of $p66^{Shc}$ led to the acquisition of a constitutively active Ras-like state. In normal primary cells, endogenously controlled native Ras is transiently activated to suppress pRB function to allow cell cycle progression (Mittnacht *et al.*, 1997; Peeper *et al.*, 1997). In contrast, expression of constitutively active oncogenic Ras mutants such as Ras(V12) in normal cells causes cell cycle arrest, an effect requiring intact pRB function (Phelps *et al.*, 1988; Serrano *et al.*, 1997). Accordingly, suppression of basal Ras activity with Ras(N17) decreased NHBE cell proliferation at baseline (Figure 4f). However, in the presence of Ras(N17), knockdown of $p66^{Shc}$ no longer suppressed proliferation, and phosphorylation of pRB was partly restored (Figures 4f and g). $p66^{Shc}$ knockdown also induced the cyclin-dependent kinase 4 inhibitor $p16^{INK4A}$, although Ras(N17) did not significantly block this induction (Figure 4g), suggesting both Ras-dependent and Ras-independent effects of $p66^{Shc}$ on pRB. These data indicate that loss of $p66^{Shc}$ may indeed leave native Ras in a dysregulated hyperactive state, leading to pRB-dependent proliferative arrest, comparable to the effects of oncogenic Ras mutations.

We further tested the effect of Ras deregulation on proliferation in the lung cancer cell lines. First, we noted that Ras(N17) did not suppress proliferation of either the LLC or SCLC cell lines, which display basal Ras hyperactivation but lack pRB (Figures 4h–j). However, upon re-expression of pRB, proliferation decreased and was restored by Ras(N17) (Figures 4h–j). These data confirm that Ras, rendered hyperactive by lack of $p66^{Shc}$, acts similarly to constitutively active oncogenic Ras in its ability to suppress proliferation through pRB, even in fully transformed cells. These data also highlight the importance of pRB loss in the development of SCLC, without which hyperactive Ras would be poorly tolerated.

$p66^{Shc}$ restrains hyperactivation of Rac1 and deactivation of RhoA downstream from Ras

Transformation triggered by oncogenic Ras requires Rac1 (Qiu *et al.*, 1995), which also mediates anchorage-dependent proliferative signals (Mettouchi *et al.*, 2001). We therefore explored a potential role for Rac1 in the $p66^{Shc}$-Ras pathway. Similar to its effect on Ras, we found that re-expression of $p66^{Shc}$ in LLC and SCLC cells reduced basal Rac1 activity; conversely, $p66^{Shc}$ knockdown in primary NHBE and human umbilical vein endothelial cells caused robust activation of Rac1 (Figures 5a and b). In NHBE cells, Rac1 hyperactivation was suppressed by Ras(N17), whereas Rac1(N17) had no effect on hyperactivation of Ras (Figures 5c and d), indicating activation of Rac1 downstream from Ras following loss of $p66^{Shc}$. In contrast, the activity of RhoA was suppressed by $p66^{Shc}$ knockdown and restored by Ras(N17) and Rac1(N17) (Figure 5e). Thus, loss of $p66^{Shc}$ blocks RhoA downstream from constitutive activation of Ras and Rac1.

Next, we placed Rac1 and RhoA in the pathways leading to control of proliferation and anoikis downstream from Ras. In LLC and SCLC cells, Rac1(N17) reversed the pRB-induced suppression of proliferation to a similar extent as Ras(N17) (Figures 5f–h). This finding suggests that hyperactive Ras suppresses growth in these malignant cells in the presence of pRB through Rac1. In contrast to Ras and Rac1, RhoA activity is suppressed in LLC and SCLC cells (Figure 1b). However, restoration of RhoA activity with RhoA(V14) had no effect on pRB-induced suppression of proliferation (Figures 5f–h). Therefore, hyperactive Ras signals bifurcate at Rac1 to suppress proliferation and anoikis through distinct pathways, with RhoA controlling anoikis and not proliferation in this context.

Lastly, to confirm that RhoA-dependent anoikis requires Ras restraint by $p66^{Shc}$, we noted that suppression of Ras hyperactivation with Ras(N17) restored anoikis in floating cultures

of LLC, H69 and H209 cells (Figures 5i–k). Furthermore, re-expression of $p66^{Shc}$, which suppresses Ras (Figure 4a), also restored anoikis; however, concomitant expression of Ras(V12) overrode this effect, blocking $p66^{Shc}$-induced anoikis in all three cell lines (Figures 5i–k). These data confirm that the effects of $p66^{Shc}$ on Ras restraint govern RhoA-dependent anoikis.

Discussion

The model that emerges from this study suggests that $p66^{Shc}$ serves as an important coordination point influencing Ras and pRB pathways (Figure 6). Given its role in initiating anchorage-sensing pathways (Ma *et al.*, 2007), we speculate that $p66^{Shc}$ normally lends attachment context to cell-fate decisions through these two pathways. Ras and pRB have complex functional interactions controlling proliferation and differentiation. Detailed genetic studies suggest that endogenous N-Ras and K-Ras interact with *Rb1* loss in mice to suppress differentiation of pituitary tumors and enhance malignant transformation (Takahashi *et al.*, 2004,2006). However, in these *Rb1* heterozygotes, N-Ras but not K-ras appears to have an opposite role in the progression of C-cell adenomas to metastatic carcinomas (Takahashi *et al.*, 2006), suggesting tissue- and isoform-specific effects of Ras. Notably, these studies suggest that in the setting of tumorigenesis following loss of pRB, Ras influences differentiation rather than proliferation. Studies using oncogenic Ras to initiate tumorigenesis generally support the role of Ras in reversing differentiation. In this context, pRB acts as a tumor suppressor by irreversibly arresting proliferation in the face of constitutively active Ras (Lundberg *et al.*, 2000); however, suppression of the pRB pathway allows oncogenic Ras to facilitate anchorage-independent growth *in vitro* and promote tumorigenesis *in vivo* despite low proliferation rates, indicating loss of differentiation as the primary effect of oncogenic Ras (Phelps *et al.*, 1988;Ho *et al.*, 2009).

Interestingly, our data suggest that in the absence of $p66^{Shc}$, native K-Ras assumes a hyperactive, deregulated state similar to oncogenic Ras. Knockdown of $p66^{Shc}$ activates pRB in normal cells, and re-expression of pRB by SCLC and LLC cells enforces this antiproliferative function of hyperactive Ras. Furthermore, in both normal and malignant cells, Ras activated by the absence of $p66^{Shc}$ acts primarily to disable anoikis rather than promote proliferation rates, again similar to the effects of oncogenic Ras on reversing differentiation. The loss of metastatic capacity upon re-expression of $p66^{Shc}$ by LLC cells effectively demonstrates the biological significance of this effect. Given its parallel effects on restoring anoikis in SCLC cells, these results suggest that $p66^{Shc}$ may act as an important metastasis suppressor.

An additional ramification of Ras deregulation may lie in the observation that oncogenic Ras mutations are rarely found in SCLC (Wistuba *et al.*, 2001). In the H69 and H209 cell lines, we demonstrate that K-Ras is constitutively hyperactivated in the absence of $p66^{Shc}$, leading to their characteristic anchorage-independent phenotype. Such basal hyperactivation would suggest that there would be little selective pressure favoring the outgrowth of cells harboring activating mutations of Ras. This interpretation is consistent with the lack of effect of oncogenic v-Ras on classic SCLC lines (Mabry *et al.*, 1988).

The mechanism by which $p66^{Shc}$ restrains Ras and thus promotes RhoA activation remains unclear. $p66^{Shc}$ is known to antagonize the proliferative effects of EGF, which work in part through $p52^{Shc}$ and therefore suggests an antagonistic relationship between the two isoforms (Migliaccio *et al.*, 1997; Okada *et al.*, 1997). The basis for the divergent effects of the two Shc isoforms on EGF/EGFR signaling is thought to be related to competition for the Sos adapter Grb2, although one anticipated effect is activation of Rac1 at the expense of Ras (Khanday *et al.*, 2006), an effect that we did not find. Furthermore, although $p66^{Shc}$ is felt to

be a negative regulator of ERK, the direct effect of $p66^{Shc}$ on Ras itself has not been studied. A more widely recognized effect of $p66^{Shc}$ is its ability to initiate oxidative stress and cell death, through translocation to the mitochondrial inner membrane and redox cycling with cytochrome *c* (Giorgio *et al.*, 2005). However, we have previously shown that $p66^{Shc}$ mutations that abrogate cytochrome *c* binding retain full capacity to initiate anoikis (Ma *et al.*, 2007), consistent with its primary effect in conferring attachment context through regulation of Ras.

We conclude that $p66^{Shc}$ normally restrains Ras from becoming hyperactivated with subsequent loss of RhoA-dependent anoikis. As in the case of oncogenic Ras, pRB is activated as a downstream failsafe consequence following loss of $p66^{Shc}$ expression, halting proliferation. In the evolution of highly metastatic tumors such as SCLC, the loss of *RB1* may allow subsequent repression of $p66^{Shc}$ with acquisition of metastatic capacity.

Materials and methods

Cloning and transduction

Human $p66^{Shc}$ (Ma *et al.*, 2007), pRB (Addgene plasmid 10720; Sellers *et al.*, 1998), HPV 16 E7 (Addgene plasmid 10720; Sellers *et al.*, 1998) and the nonbinding E7 deletion mutant E7(Δ21–24) (Addgene plasmid 13687; Gonzalez *et al.*, 2001) were ligated into the lentiviral shuttle pCCL.PPT.hPGK.IRES. eGFP/pre (gift of Philipp Scherer), and were used to produce lentivirus in Phoenix-293 cells with the packaging plasmids pMD2.BSBG, pMDLg/pRRE and pRSV-REV. Oligos encoding shRNA specific for the p66 isoform of Shc were ligated into pSUPER.retro.puro, and the fragment containing the H1 promoter and hairpin sequences was subcloned into the lentiviral shuttle pCCL.PPT.hPGK. GFP.Wpre and used to produce lentivirus as above. shRNA(1) targets coding nucleotides 42–60 of $p66^{Shc}$ and shRNA(2) targets nucleotides 252–270. $p66^{Shc}$ or p66(S36E) (Ma *et al.*, 2007) were subcloned into the retroviral vector PINCO (gift of Pier Guiseppe Pelicci), which contains a separate EGFP cassette, and retrovirus was produced from the Phoenix-293 feeder line. Adenovirus containing Myc-Rac1(N17) was a gift of Kaikobad Irani, and adenoviruses containing RhoA(N19) and RhoA(V14) were gifts of Christopher Chen. Adenoviruses containing Ras(V12) and Ras(N17) were obtained from Vector Biolabs (Philadelphia, PA, USA).

Immunoblots

The following antibodies were used: Shc and Rac1 (BD Biosciences, San Jose, CA, USA); pRB and pS780-pRB (Cell Signaling, Danvers, MA, USA); RhoA, $p16^{INK4A}$, pan-Ras and N-Ras (Santa Cruz, Santa Cruz, CA, USA), actin (Millipore, Billerica, MA, USA); and H-Ras and K-Ras (Abgent, San Diego, CA, USA).

GTPase pulldown assays

Active RhoA was assessed by pulldown using rhotekin-RBD-GST (Ma *et al.*, 2007). Active Ras was assessed with pulldown using Raf-RBD-GST (Wu *et al.*, 2007). Isoform-specific activation of Ras was assessed using the same pulldown technique followed by immunoblot with isoform-specific antibodies. Rac1 activity was assessed by pulldown using PBD-GST (Wu *et al.*, 2007). Two-thirds of each sample was used for the pulldown assay and the remaining sample acetone-precipitated for assessment of total GTPase.

Cell culture

Phoenix-293 and Lewis lung carcinoma (LLC1) cells were obtained from American Type Culture Collection (Manassas, VA, USA). NHBE and human umbilical vein endothelial

cells were from Lonza (Basel, Switzerland), and were routinely used at passages four and five. H209 and H69 SCLC cells were gifts of John Minna.

LLC metastasis

Retrovirus containing $p66^{Shc}$ or p66(S36E) were used to infect LLC cells, which were subsequently treated with 1 μg/ml puromycin. Puromycin-resistant cells were then selected by cell sorting for GFP expression (FACS Vantage, BD). 5×10^5 cells of each group in 100 μl saline were injected into the tail vein of 6-week-old female C57BL/6 mice. At 45 days following injection, surviving mice were killed. GFP-positive lung metastases were observed with the CRi MaestroTM imaging system (CRi, Woburn, MA, USA). Lungs were blotted, weighed and inflation fixed with formalin at 15cm H_2O pressure.

Cell death and proliferation assays

Cells were plated on either cell culture-treated or low-attachment 24-well plates (Corning, Wilkes Barre, PA, USA) for 16 h. Cell death was assessed as DNA fragmentation using a cell death enzyme-linked immunosorbent assay (Roche, Basel, Switzerland). Absorbance was normalized for cell number. Proliferation was assessed using a 5-bromodeoxyuridine incorporation enzyme-linked immunosorbent assay (Roche), again normalizing for cell number.

Statistical analysis

Statistical analysis of cell death and proliferation end points was performed using analysis of variance with Tukey's *post hoc* testing. Kapaln–Meier survival curves were analyzed with a two-tailed log-rank test.

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References

Attwell S, Roskelley C, Dedhar S. The integrin-linked kinase (ILK) suppresses anoikis. Oncogene 2000;19:3811–3815. [PubMed: 10949937]

Douma S, Van Laar T, Zevenhoven J, Meuwissen R, Van Garderen E, Peeper DS. Suppression of anoikis and induction of metastasis by the neurotrophic receptor TrkB. Nature 2004;430:1034–1039. [PubMed: 15329723]

Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 1994;124:619–626. [PubMed: 8106557]

Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY. Control of adhesion-dependent cell survival by focal adhesion kinase. J Cell Biol 1996;134:793–799. [PubMed: 8707856]

Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, et al. Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. Cell 2005;122:221–233. [PubMed: 16051147]

Gonzalez SL, Stremlau M, He X, Basile JR, Munger K. Degradation of the retinoblastoma tumor suppressor by the human papillomavirus type 16 E7 oncoprotein is important for functional inactivation and is separable from proteasomal degradation of E7. J Virol 2001;75:7583–7591. [PubMed: 11462030]

Grignani F, Kinsella T, Mencarelli A, Valtieri M, Riganelli D, Lanfrancone L, et al. High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. Cancer Res 1998;58:14–19. [PubMed: 9426049]

Ho VM, Schaffer BE, Karnezis AN, Park KS, Sage J. The retinoblastoma gene Rb and its family member p130 suppress lung adenocarcinoma induced by oncogenic K-Ras. Oncogene 2009;28:1393–1399. [PubMed: 19151761]

Khanday FA, Santhanam L, Kasuno K, Yamamori T, Naqvi A, Dericco J, et al. Sos-mediated activation of rac1 by p66shc. J Cell Biol 2006;172:817–822. [PubMed: 16520382]

Lundberg AS, Hahn WC, Gupta P, Weinberg RA. Genes involved in senescence and immortalization. Curr Opin Cell Biol 2000;12:705–709. [PubMed: 11063935]

Ma Z, Myers DP, Wu RF, Nwariaku FE, Terada LS. p66Shc mediates anoikis through RhoA. J Cell Biol 2007;179:23–31. [PubMed: 17908916]

Mabry M, Nakagawa T, Nelkin BD, McDowell E, Gesell M, Eggleston JC, et al. v-Ha-ras oncogene insertion: a model for tumor progression of human small cell lung cancer. Proc Natl Acad Sci USA 1988;85:6523–6527. [PubMed: 2842776]

McFall A, Ulku A, Lambert QT, Kusa A, Rogers-Graham K, Der CJ. Oncogenic Ras blocks anoikis by activation of a novel effector pathway independent of phosphatidylinositol 3-kinase. Mol Cell Biol 2001;21:5488–5499. [PubMed: 11463831]

Mettouchi A, Klein S, Guo W, Lopez-Lago M, Lemichez E, Westwick JK, et al. Integrin-specific activation of Rac controls progression through the G(1) phase of the cell cycle. Mol Cell 2001;8:115–127. [PubMed: 11511365]

Migliaccio E, Mele S, Salcini AE, Pelicci G, Lai KM, Superti-Furga G, et al. Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. EMBO J 1997;16:706–716. [PubMed: 9049300]

Mittnacht S, Paterson H, Olson MF, Marshall CJ. Ras signalling is required for inactivation of the tumour suppressor pRb cell-cycle control protein. Curr Biol 1997;7:219–221. [PubMed: 9395436]

Okada S, Kao AW, Ceresa BP, Blaikie P, Margolis B, Pessin JE. The 66-kDa Shc isoform is a negative regulator of the epidermal growth factor-stimulated mitogen-activated protein kinase pathway. J Biol Chem 1997;272:28042–28049. [PubMed: 9346957]

Peeper DS, Upton TM, Ladha MH, Neuman E, Zalvide J, Bernards R, et al. Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. Nature 1997;386:177–181. [PubMed: 9062190]

Phelps WC, Yee CL, Munger K, Howley PM. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. Cell 1988;53:539–547. [PubMed: 2836062]

Qiu RG, Chen J, Kirn D, McCormick F, Symons M. An essential role for Rac in Ras transformation. Nature 1995;374:457–459. [PubMed: 7700355]

Sellers WR, Novitch BG, Miyake S, Heith A, Otterson GA, Kaye FJ, et al. Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth. Genes Dev 1998;12:95–106. [PubMed: 9420334]

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997;88:593–602. [PubMed: 9054499]

Takahashi C, Contreras B, Bronson RT, Loda M, Ewen ME. Genetic interaction between Rb and K-ras in the control of differentiation and tumor suppression. Mol Cell Biol 2004;24:10406–10415. [PubMed: 15542848]

Takahashi C, Contreras B, Iwanaga T, Takegami Y, Bakker A, Bronson RT, et al. Nras loss induces metastatic conversion of Rb1-deficient neuroendocrine thyroid tumor. Nat Genet 2006;38:118–123. [PubMed: 16369533]

Wistuba II, Gazdar AF, Minna JD. Molecular genetics of small cell lung carcinoma. Semin Oncol 2001;28:3–13. [PubMed: 11479891]

Wu RF, Ma Z, Myers DP, Terada LS. HIV-1 Tat activates dual Nox pathways leading to independent activation of ERK and JNK MAP kinases. J Biol Chem 2007;282:37412–37419. [PubMed: 17940286]

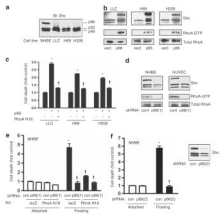

Figure 1.

p66^{Shc} promotes anoikis through RhoA. (**a**) Immunoblot for Shc demonstrates expression of three Shc isoforms by normal human bronchial epithelium (NHBE) cells but loss of p66^{Shc} expression by Lewis lung carcinoma (LLC) and SCLC (H69 and H209) cell lines. (**b**) LLC, H69 and H209 cells were transduced with p66^{Shc}. The top panels demonstrate Shc isoform expression. Active RhoA was assessed by pulldown and was shown to consistently increase following expression of p66^{Shc}. (**c**) LLC, H69 and H209 cells were grown in low attachment plates after lentiviral transduction with p66^{Shc} or empty vector, and adenoviral transduction with dominant-negative RhoA(N19) or lacZ (control). After 16 h, cell death was assessed. Mean ± s.e.m. of four determinations is shown. *$P < 0.001$ compared with respective control, †$P < 0.001$ compared with p66^{Shc} alone. (**d**) p66^{Shc} was knocked down with shRNA in NHBE cells and human umbilical vein endothelial cells (HUVECs). The top panels demonstrate knockdown effect. The bottom panels show that p66^{Shc} knockdown completely suppressed RhoA activity. (**e**) NHBE cells were transduced with the indicated shRNA by lentivirus and RhoA(N19) or lacZ by adenovirus, and allowed to adhere or forced to float for 16 h. Cell death was measured as above. Mean ± s.e.m. of four determinations is shown. *$P < 0.001$ compared with attached control, †$P < 0.001$ compared with floating control. (**f**) NHBE cells were transduced with a second shRNA against p66^{Shc} (shRNA(2)) and cell death measured as above. Inset shows selective effect of shRNA(2) for p66^{Shc}. Mean ± s.e.m. of four determinations is shown. *$P < 0.001$ compared with attached control, †$P < 0.001$ compared with floating control.

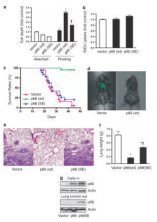

Figure 2.

p66^{Shc} suppresses lung metastasis *in vivo*. (**a**) LLC cells stably expressing p66^{Shc} or p66(S36E) were plated on normal or low attachment plates for 16 h and cell death assessed. Expression of wild-type p66^{Shc} increased detachment-induced cell death (*$P < 0.001$ from vector control), whereas p66(S36E) had a reduced effect ($^{\dagger}P < 0.001$ from p66^{Shc} floating). Mean ± s.e.m. of four determinations is shown. (**b**) 5-bromodeoxyuridine (BrDU) uptake of attached LLC cells expressing p66^{Shc} (p66 wt) or p66(S36E) was not different from vector control. (**c**) LLC cells expressing p66^{Shc} or p66(S36E)were injected into the tail vein of 6-week-old female C57BL/6 mice. Kaplan–Meier survival curve is shown; survival between groups was different, $P < 0.0001$. $n = 19$ (vector), $n = 21$ (p66 wt) and $n = 19$ (p66(SE)). (**d**) Representative mice receiving vector-LLC or p66-LLC cells. Multiple GFP-positive lung metastases are apparent in the former but not in the latter group. (**e**) Representative hematoxylin and eosin stain of lungs from mice injected with vector control, p66^{Shc} or p66(S36E)-expressing LLC cells. Vector control and p66(S36E) lungs demonstrated dense interstitial replacement with tumor with perivascular tumor cuffing. (**f**) Wet lung weights were assessed, reflecting tumor burden. *$P < 0.01$ from vector control, $^{\dagger}P < 0.01$ from p66 (wt). (**g**) Immunoblot showing p66^{Shc} expression of cells injected into animals (top panel). Lung tumors were dissected, pooled and immunoblotted for p66^{Shc} from the single mouse in the p66^{Shc} group that had metastatic tumors (middle lane), and representative tumors extracted from lungs of mice receiving LLC cells with empty vector (left lane) or p66(S36E) (right lane).

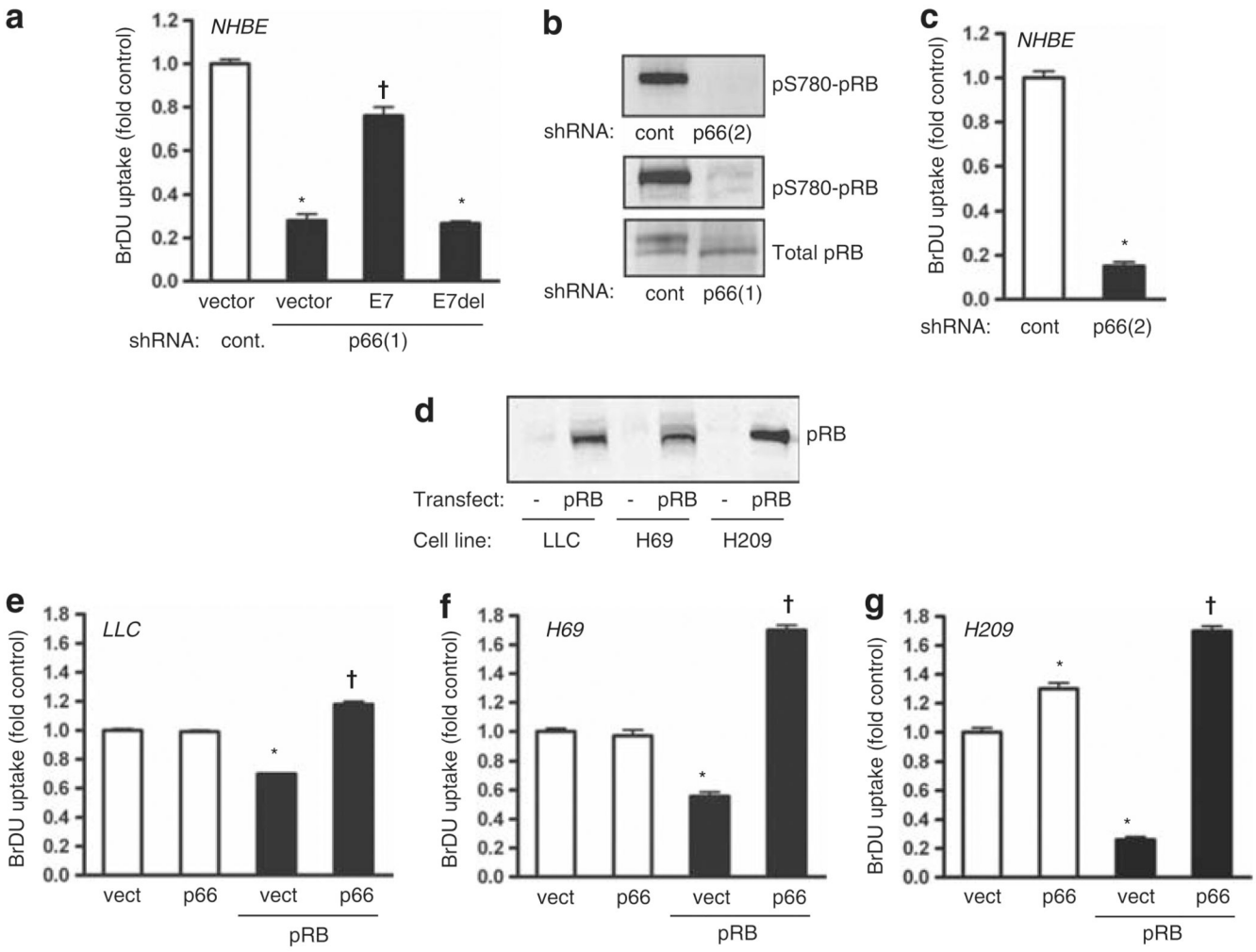

Figure 3.

p66^{Shc} suppresses proliferation through pRB. (**a**) NHBE cells were transduced with either HPV16 E7 or its nonbinding deletion mutant E7(Δ21–24), and proliferation assessed. Knockdown of p66^{Shc} decreased 5-bromodeoxyuridine (BrDU) uptake (*$P < 0.001$ from vector control), and E7 but not E7(Δ21–24) restored proliferation ($^{\dagger}P < 0.01$ from p66^{Shc} knockdown alone). Mean ± s.e.m. of six determinations is shown. (**b**) NHBE cells were transduced with control or p66^{Shc} shRNA and immunoblotted for total and phospho-pRB, demonstrating hypophosphorylation of pRB following p66^{Shc} knockdown. (**c**) Proliferation was assessed following transduction with indicated shRNAs. Mean ± s.e.m. of six determinations, *$P < 0.001$ from vector control. (**d**) Immunoblot for total pRB of the indicated cell lines before and after lentiviral delivery of pRB. (**e–g**) Lentivirus was used to transduce p66^{Shc} and pRB as indicated. pRB decreased BrDU incorporation in LLC, H69 and H209 cells (*$P < 0.001$ from vector control) whereas p66^{Shc} restored proliferation above baseline ($^{\dagger}P < 0.01$ from pRB alone). Mean ± s.e.m. of six determinations is shown.

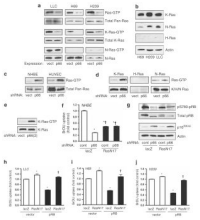

Figure 4.

p66^{Shc} restrains Ras hyperactivation in malignant and normal cells. (**a**) LLC, H69 and H209 tumor cell lines were transduced with lentivirus as indicated and active Ras was assessed by pulldown. Expression of p66^{Shc} markedly suppressed Ras activation as assessed by immunoblot for pan-Ras (top panels) and K-Ras (middle panels) but not N-Ras (bottom panels). (**b**) Immunoblots of SCLC and LLC cells for different Ras isoforms, with actin loading control. (**c**) Knockdown of p66^{Shc} in primary NHBE cells and human umbilical vein endothelial cells (HUVEC) caused activation of Ras. (**d**) p66^{Shc} knockdown caused robust activation of K-Ras but not H-Ras or N-Ras in NHBE cells. (**e**) Similar conditions as in (**d**) except with shRNA(2) against p66^{Shc}. (**f**) In NHBE cells, 5-bromodeoxyuridine (BrDU) uptake was assessed following knockdown of p66^{Shc} and/or expression of Ras(N17). Mean ± s.e.m. of six determinations is shown, $^{*}P < 0.001$ from lacZ control, $^{\dagger}P < 0.001$ from lacZ/p66^{Shc} knockdown. (**g**) Immunoblot for phosphorylated and total pRB and p16^{INK4A} of NHBE cells treated as indicated. (**h–j**) BrDU uptake was measured in LLC, H69 and H209 cells after expression of pRB and/or Ras (N17). pRB decreased BrDU uptake ($^{*}P < 0.001$ from lacZ/vector control), whereas Ras(N17) restored proliferation ($^{\dagger}P < 0.001$ from lacZ/pRB, $P > 0.05$ from lacZ/vector control). Mean ± s.e.m. of six determinations is shown.

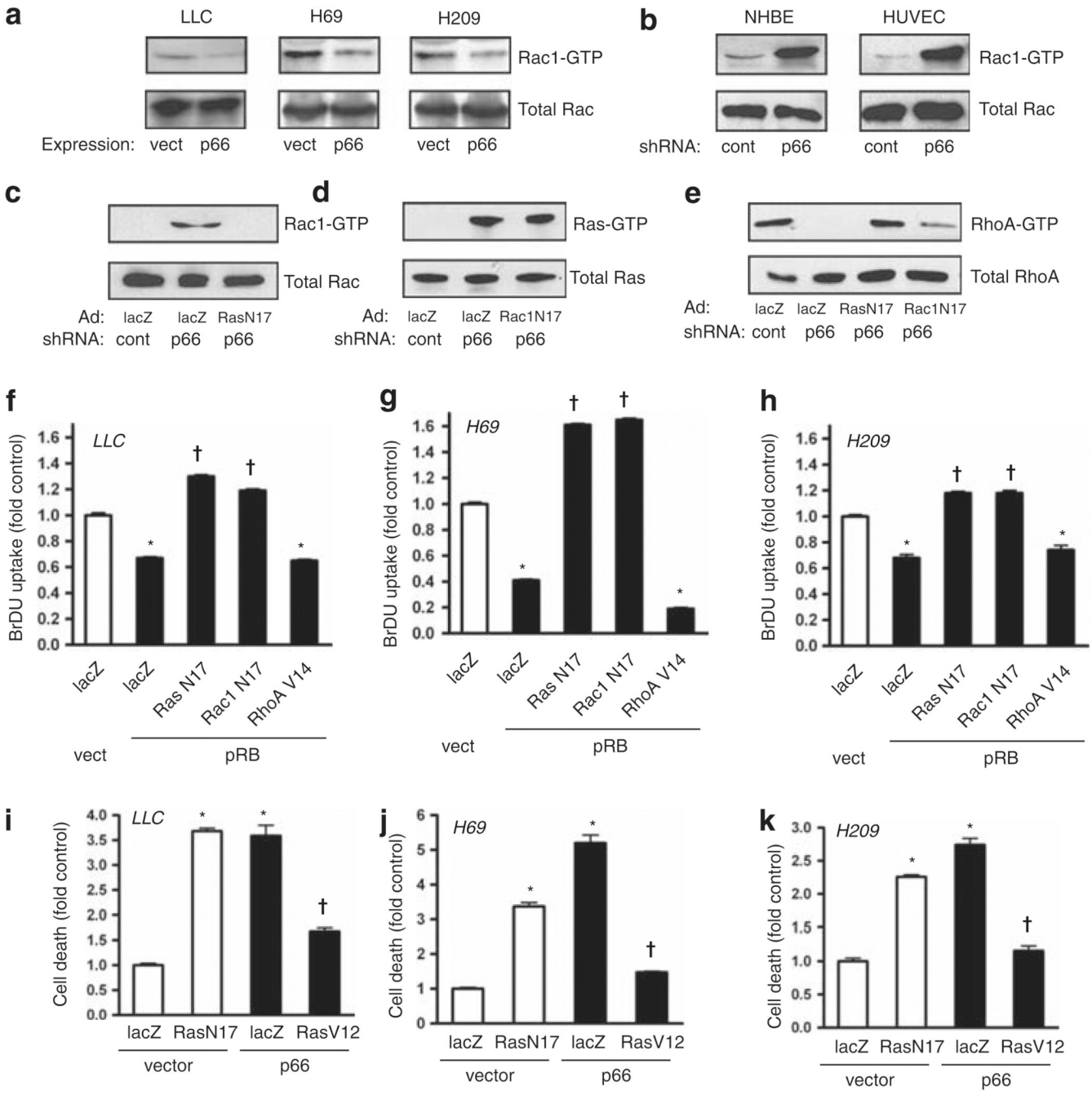

Figure 5.

Loss of p66^{Shc} suppresses RhoA through Ras and Rac1 activation. (**a**) Re-expression of p66^{Shc} through lentiviral delivery suppressed active Rac1, assessed by pulldown. (**b**) Knockdown of p66^{Shc} by shRNA caused intense activation of Rac1 in NHBE cells and human umbilical vein endothelial cells (HUVECs). (**c–e**) Ras(N17) or Rac1(N17) were expressed and p66^{Shc} knocked down as indicated in NHBE cells, and active Rac1, Ras and RhoA were assessed by pulldown. (**f–h**) Dominant negatives Ras(N17) and Rac1(N17) and constitutively active RhoA(V14) were delivered by adenovirus; pRB was expressed by lentiviral delivery in LLC, H69 and H209 cells. pRB suppressed BrDU uptake (*$P < 0.001$ from lacZ/vector control), whereas both Ras(N17) and Rac1(N17) restored proliferation

above baseline ($^{\dagger}P < 0.001$ from lacZ/pRB). Active RhoA(V14) did not rescue proliferation from pRB. Mean ± s.e.m. of six determinations is shown. (**i–k**) LLC, H69 and H209 cells were grown in low attachment plates following transduction as indicated. DNA fragmentation was assessed 16 h later. Suppression of endogenous Ras with Ras(N17) caused death in floating cells for all three cell lines to levels comparable to those caused by $p66^{Shc}$ expression ($^{*}P < 0.001$ from lacZ/vector control). In contrast, active Ras(V12) rescued all three cells from $p66^{Shc}$-induced anoikis ($^{\dagger}P < 0.001$ from respective lacZ/p66 groups). Mean ± s.e.m. of four determinations is shown.

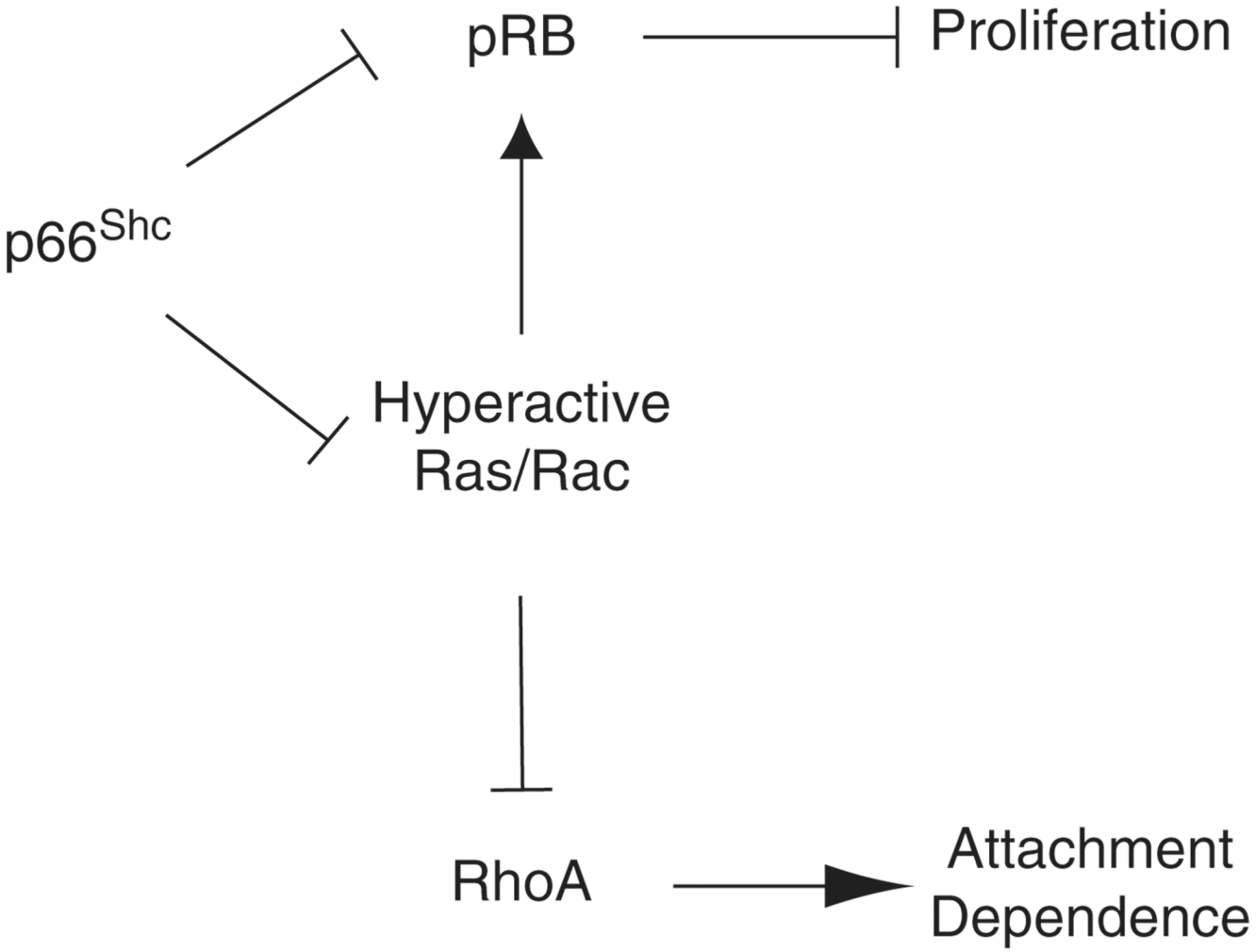

Figure 6.
Schematic indicating relationship of p66^{Shc} to proliferative and anoikis pathways through Ras and pRB, consistent with findings in both normal primary bronchial epithelium and malignant lung cancer lines studied. p66^{Shc} restrains Ras and Rac1 from hyperactivation. Signals bifurcate beyond Rac1 to control proliferation through pRB and attachment sensing through RhoA. Loss of anchorage context following loss of p66^{Shc} would allow survival while detached but suppress proliferation through pRB. The diagram includes possible Ras-independent effect of p66^{Shc} on pRB suppression.