

PKC δ regulates integrin $\alpha_v\beta_3$ expression and transformed growth of K-ras dependent lung cancer cells

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ABSTRACT

We have previously shown that Protein Kinase C delta (PKC δ) functions as a tumor promoter in non-small cell lung cancer (NSCLC), specifically in the context of K-ras addiction. Here we define a novel PKC δ \rightarrow integrin $\alpha_v\beta_3$ \rightarrow Extracellular signal-Regulated Kinase (ERK) pathway that regulates the transformed growth of K-ras dependent NSCLC cells. To explore how PKC δ regulates tumorigenesis, we performed mRNA expression analysis in four KRAS mutant NSCLC cell lines that stably express scrambled shRNA or a PKC δ targeted shRNA. Analysis of PKC δ -dependent mRNA expression identified 3183 regulated genes, 210 of which were specifically regulated in K-ras dependent cells. Genes that regulate extracellular matrix and focal adhesion pathways were most highly represented in this later group. In particular, expression of the integrin pair, $\alpha_v\beta_3$, was specifically reduced in K-ras dependent cells with depletion of PKC δ , and correlated with reduced ERK activation and reduced transformed growth as assayed by clonogenic survival. Re-expression of PKC δ restored *ITGAV* and *ITGB3* mRNA expression, ERK activation and transformed growth, and this could be blocked by pretreatment with a $\alpha_v\beta_3$ function-blocking antibody, demonstrating a requirement for integrin $\alpha_v\beta_3$ downstream of PKC δ . Similarly, expression of integrin α_v restored ERK activation and transformed growth in PKC δ depleted cells, and this could also be inhibited by pretreatment with PD98059. Our studies demonstrate an essential role for $\alpha_v\beta_3$ and ERK signaling downstream of PKC δ in regulating the survival of K-ras dependent NSCLC cells, and identify PKC δ as a novel therapeutic target for the subset of NSCLC patients with K-ras dependent tumors.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death in men and women, with the majority of those tumors diagnosed as non-small cell lung cancer (NSCLC) [1, 2]. NSCLC may be further categorized by the driver mutations found in certain subtypes. Oncogenic KRAS mutations are found in approximately 25% of adenocarcinomas, the largest sub-type of NSCLC [3]. Tumors harboring oncogenic KRAS mutations, regardless of tumor site, have poor clinical outcomes. Recently, several groups have reported that a subset of KRAS mutant tumors are fully reliant on the KRAS oncogene for their

survival, i.e., are K-ras dependent, while others have lost their addiction to K-ras and are presumably dependent on alternative survival pathways [4]. Understanding the signaling pathways that regulate tumorigenesis in these K-ras dependent cancer cells will be important for the development of effective therapies for patients with these treatment refractive tumors.

The PKC family is comprised of 10 serine/threonine kinases that have been implicated in numerous biological processes, including proliferation, the immune response, survival, and apoptosis [5]. PKC ϵ and PKC ι/λ are most strongly associated with human cancer, while the function of other isoforms in cancer, including PKC δ , appears to

be context dependent [6]. Studies in PKC δ knock-out mice have confirmed a role for this kinase in cell death in response to irradiation [7] and during mammary gland involution [8]. *In vitro*, depletion or inhibition of PKC δ results in resistance to multiple apoptotic stimuli [8-10]. While most non-transformed cells use PKC δ for apoptotic signaling, in many cancer cells these pathways are disabled. This may underlie the somewhat paradoxical observation that in certain oncogenic contexts PKC δ appears to be required for cancer cell growth. For example, studies from our lab have shown that PKC δ is required for tumorigenesis driven by oncogenic K-ras [11] and that PKC δ regulates proliferation of Her2/neu driven tumors *in vivo* and in human breast cancer cells *in vitro* [12]. PKC δ has also been shown to promote tumor progression of human pancreatic cancer, to function as a tumor promoter in a mouse model of skin cancer, and to negatively regulate the proliferation and survival of cancer stem cells [13-15].

To understand the mechanism by which PKC δ functions as a tumor promoter, we analyzed PKC δ regulated genes in K-ras dependent and independent NSCLC cells. Our studies identify focal adhesion signaling and extracellular matrix (ECM) genes as differentially regulated in K-ras dependent versus K-ras independent NSCLC cells. These include the integrin genes, *ITGAV* and *ITGB3* that code for the heterodimer, integrin $\alpha_v\beta_3$. Increased expression of integrin $\alpha_v\beta_3$ correlates with a poor prognosis in some human tumors [16]. Integrin $\alpha_v\beta_3$ acts as a receptor for ECM ligands, including fibronectin and vitronectin, and is a well-established regulator of invasion and anchorage-independent growth *in vitro* [17, 18]. Integrin $\alpha_v\beta_3$ can also have ligand-independent functions in tumor cells [18] and recent studies show that un-ligated integrin $\alpha_v\beta_3$ can drive cancer cell stemness and drug resistance through activation of K-ras and RalB [19]. Our studies describe a novel PKC δ ->integrin $\alpha_v\beta_3$ -> Extracellular signal-Regulated Kinase (ERK) pathway that is important for regulation of transformed growth specifically in K-ras dependent NSCLC cells, and suggest that perturbation of this pathway may be a novel therapeutic strategy for the subset of NSCLC patients with K-ras dependent tumors.

RESULTS

Expression profiling of genes regulated by PKC δ in K-ras mutant NSCLC cells

We have previously shown that PKC δ is required for tumorigenesis driven by oncogenic K-ras and for the survival of human NSCLC cell lines that are dependent on K-ras [11]. To further understand the function of PKC δ in the context of oncogenic K-ras we sought to identify

genes and functional pathways whose expression is specifically regulated by PKC δ . Transcriptional profiling using Affymetrix GeneChip human genome arrays was performed in two K-ras dependent (H2009 and H441) and two K-ras independent (A549 and H460) NSCLC cell lines that stably express shRNA targeting either the coding region of PKC δ (δ 193) or a scrambled non-targeting sequence (δ scr). Using a 1.25 fold cut-off, our analysis revealed 3183 genes that show a statistically significant change in gene expression in all cell lines with depletion of PKC δ regardless of their K-ras dependency status. Analysis of gene expression in H2009 and H441 cells revealed 210 genes significantly regulated in both cell lines; 116 genes were down-regulated and 94 genes were up-regulated with depletion of PKC δ (Table S1). In K-ras independent cells, 124 genes were significantly regulated in both cell lines; 77 genes were down-regulated, while 47 genes were up-regulated with depletion of PKC δ (Table S1). Notably, 23 of the 116 genes that were down regulated in K-ras dependent cells were also down regulated in K-ras independent cells. Common down-regulated genes reflect the diverse biological functions of PKC δ and include *RAB23*, a small GTPase in the Ras superfamily; sorting nexin-27 (*SNX-27*); the metabolic enzymes adenosine deaminase (*ADA*) and galactosylceramidase (*GALC*); diacylglycerol kinase alpha (*DGKA*) which regulates diacylglycerol levels and PKC activation at membranes; the cytokine receptors, *IL6ST* and *LIFR*; and *BNIP3L*, an inducer of apoptosis. No up-regulated genes were shared between K-ras dependent and independent cell lines.

To gain insight into the biological pathways controlled by PKC δ in NSCLC, we evaluated the RNA expression profiles of δ scr and δ 193 expressing NSCLC cells utilizing the KEGG pathway analysis tool within NIH DAVID v6.7 [20-22]. Seventeen KEGG pathways were identified as significantly regulated by PKC δ regardless of K-ras dependency status (Table 1). The top three KEGG pathways identified across all four cell lines were: *Pathways in cancer*, *MAPK signaling*, and *Focal adhesion* (Table 1). In addition, a large number of metabolic pathways were also identified as regulated by PKC δ , including pathways important for glutathione and O-Glycan synthesis, and for amino acid, nitrogen and tryptophan metabolism (Table 1). The percentage of genes whose expression increased or decreased with depletion of PKC δ for each KEGG pathway is shown graphically in Figure 1. For KEGG pathways involved in cancer and cell signaling, the majority of genes showed decreased expression upon depletion of PKC δ . In contrast, the expression of genes in KEGG pathways involved in metabolic processes typically increased upon depletion of PKC δ .

Six KEGG pathways were significantly enriched in PKC δ depleted K-ras dependent H2009 and H441 cells (Table 2). These include genes whose products regulate ECM-receptor interaction such as *RELN*, *TNC* and *ZYX*

(increased with PKC δ depletion), and *TNXB*, *ITGAV*, *ITGA3* and *ITGB3* (decreased with PKC δ depletion), and genes that regulate cell survival and proliferation (*TP53*, *AKT3*, *MYC* and *NRAS*). KEGG pathways significantly enriched in K-ras independent cell lines A549 and H460 are also listed in Table 2. The majority of genes in these pathways encode components of receptor signaling pathways and their ligands including *ERBB2*, *JAK1*, *IL20RB*, *TLR3* and *LIFR*. Of note, the KEGG pathway *Chronic myeloid leukemia*, is enriched in both K-ras dependent and independent NSCLC cells, however, with the exception of *NRAS*, the specific genes regulated in this pathway are unique for each subtype.

PKC δ regulates integrin gene expression in K-ras dependent NSCLC cells

Further examination of gene expression changes in the KEGG pathways *Focal adhesion* and *ECM-receptor interaction* reveals enrichment in integrins, ECM proteins, and downstream signaling enzymes. We next validated these genes by qRT-PCR (Table S2). From this data set, we identified integrin genes whose expression was differentially regulated by PKC δ , based on K-ras dependency status. The products of two differentially regulated integrins, *ITGAV* and *ITGB3*, form the $\alpha_v\beta_3$ heterodimer, an integrin pair previously shown to regulate tumor cell survival and tumor metastasis in a ligand-independent fashion [17]. We confirmed the differential expression of *ITGAV* and *ITGB3* mRNA using a panel of four K-ras dependent NSCLC cell lines (H2009,

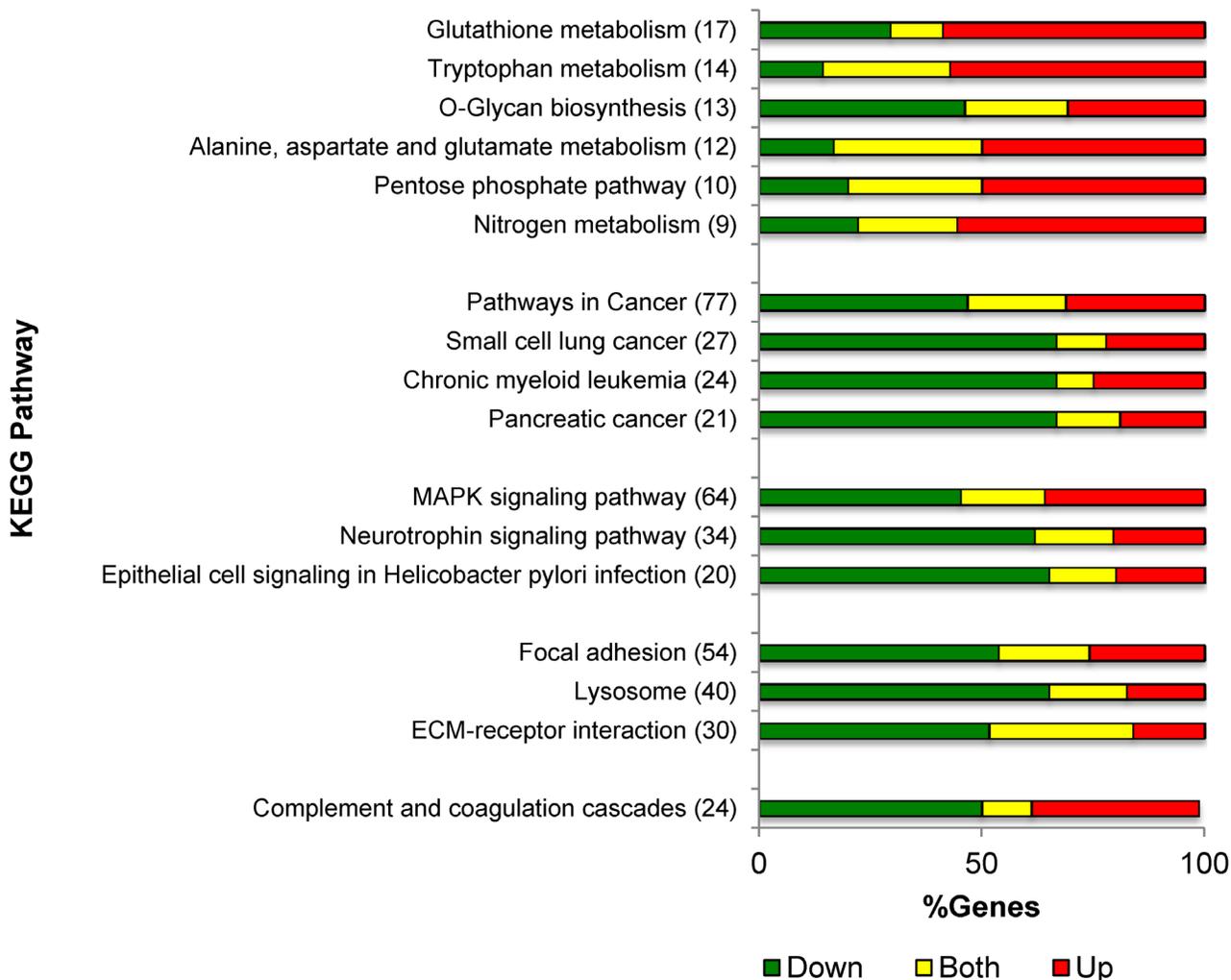


Figure 1: PKC δ regulation of gene expression pathways. Distribution of gene expression changes by KEGG pathway for genes regulated by PKC δ across all four cell lines. Shown is the number of genes regulated in each pathway, and the percent of genes with increased expression (red), decreased expression (green) or both increased and decreased expression (yellow) with depletion of PKC δ for each conserved KEGG pathway from Table 1.

Table 1: KEGG Pathways regulated by PKC δ in K-ras mutant NSCLC cells regardless of K-ras dependency status

KEGG Pathway	Gene number	p-value
Pathways in cancer	77	0.0182
MAPK signaling	64	0.0208
Focal Adhesion	54	0.0035
Lysosome	40	0.0001
Neurotrophin signaling	34	0.0164
ECM-receptor interaction	31	0.0003
Small cell lung cancer	27	0.0041
Complement and coagulation cascades	24	0.0024
Chronic myeloid leukemia	24	0.0075
Pancreatic cancer	21	0.0355
Epithelial cell signaling in Helicobacter pylori	20	0.0376
Glutathione metabolism	17	0.0157
Tryptophan metabolism	14	0.0246
O-Glycan biosynthesis	13	0.0051
Alanine, aspartate and glutamate metabolism	12	0.0194
Pentose phosphate	10	0.0302
Nitrogen metabolism	9	0.0489

All statistically significant ($p < 0.05$) KEGG pathways shown.

SW900, H358 and H441) and two K-ras independent cell lines (A549 and H460). Up to three unique shRNAs were used to deplete PKC δ , including δ 193 and δ 203 that target the coding region, and δ 625 that targets the 3' UTR of the PKC δ mRNA (Figure 2C). Analysis of *ITGAV* mRNA showed a significant decrease in K-ras dependent NSCLC cells with PKC δ depletion, while no consistent change was observed in A549 or H460 K-ras independent NSCLC cells depleted of PKC δ (Figure 2A). Analysis of *ITGB3* mRNA expression revealed a similar pattern (Figure 2B). To determine if changes in mRNA expression correlate with changes in integrin $\alpha_v\beta_3$ expression at the cell surface, we used an antibody that specifically recognizes the integrin $\alpha_v\beta_3$ dimer. Consistent with mRNA expression, depletion of PKC δ led to a decrease in cell surface integrin $\alpha_v\beta_3$ in K-ras dependent H2009, H358 and SW900 cells, relative to cells expressing δ scr shRNA (Figures 2D, 2E and 2F). Stable depletion of PKC δ in the K-ras independent cell line A549 also resulted in a slight but consistent decrease in cell surface expression of integrin $\alpha_v\beta_3$ (Figure 2G) even though no change in mRNA expression was observed (Figure 2A and 2B), suggesting that PKC δ may also contribute to mobilization

of integrin $\alpha_v\beta_3$ to the cell surface.

To confirm that PKC δ regulates *ITGAV* and *ITGB3* gene expression, we rescued PKC δ expression in H2009 δ 625 cells by adenoviral transduction of GFP-tagged PKC δ (Ad-GFP-PKC δ) or Ad-GFP (Figure 3A). Re-expression of PKC δ was verified by qRT-PCR (Figure 3A, left). Rescue of PKC δ resulted in increased expression of both *ITGAV* and *ITGB3* mRNA (Figure 3A, middle and right). To determine if expression of *ITGAV* and *ITGB3* are coordinately regulated, we restored integrin α_v expression by transient transfection of pLenti-*ITGAV* and assayed *ITGB3* mRNA. Surprisingly, expression of *ITGAV* cDNA restored expression of both *ITGAV* and *ITGB3* mRNA expression (Figure 3B, middle and right). This suggests that reduced expression of *ITGB3* in PKC δ depleted cells may be a consequence of reduced expression of *ITGAV* mRNA. However, re-expression of integrin α_v also increased *PRKCD* mRNA (Figure 3B, left) and protein expression (Figure 6E), which could contribute to the increase in *ITGB3* mRNA expression observed. In contrast, expression of *ITGB3* cDNA (Figure 3C, right) had no effect on *ITGAV* or *PRKCD* mRNA levels (Figure 3C, left and middle).

PKC δ regulates transformed growth of K-ras independent NSCLC cells

Integrin $\alpha_v\beta_3$ expression is associated with metastasis in human tumors, presumably through its ability to transmit survival signals in anchorage-independent environments [18]. To determine if decreased expression of integrin $\alpha_v\beta_3$ upon depletion of PKC δ correlates with reduced transformed growth, we assayed the colony forming ability of NSCLC cells that were detached and re-plated immediately into a clonogenic survival assay ("adherent culture", Figure 4A), and of cells that were cultured for 24 hours on poly-HEMA coated plates to prevent adhesion, and then re-plated into a clonogenic survival assay ("suspension culture", Figure 4C). Clonogenic growth under both conditions was significantly reduced with PKC δ depletion in three K-ras dependent NSCLC cell lines (H2009, SW900, and H358), while two K-ras independent cell lines (H460 and A549) showed no decrease with PKC δ depletion. The differences in clonal survival observed between these subpopulations of NSCLC cells were not the result of decreased cell viability (Figure 4B and 4D). Similarly, no significant difference in apoptosis was detected between δ scr and δ 193 cells under either culture condition (Figure 4E and 4F). Thus, it is likely that the reduced transformed growth observed in K-ras dependent cells with depletion of PKC δ reflects a diminished ability to establish colonies under clonogenic culture conditions.

As both PKC δ and integrin $\alpha_v\beta_3$ can function as upstream activators of ERK [11], a pathway essential for survival when cells lose their attachment to the ECM

[23], we explored the contribution of ERK activation to the transformed growth of K-ras mutant NSCLC cells. As shown in Figure 5A, following suspension culture for 24 hours, ERK activation was lower in H2009 and H358 cells depleted of PKC δ (δ 193 and δ 203) compared to δ scr cells, while neither A549 δ 193 and δ 203 or H460 δ 193 cells showed reduced ERK activation. In some experiments a slight decrease in ERK activation was seen in H460 δ 203 cells, however this reduction was much less than that seen in either H2009 or H358 δ 193 or δ 203 cells. The value under the blot indicates the ratio of pERK/ERK for each cell line (Figure 5A). Activation of ERK in adherent H2009 δ 193 cells, and following suspension for 4 hour, was also reduced compared to H2009 δ scr cells (Figure 5B). To address the contribution of ERK activity to clonogenic survival of K-ras mutant NSCLC

cells, we pre-treated A549 or H2009 cells with the MEK inhibitor, PD98059, either during plating and clonogenic growth (Figure 5C and 5D, "P"), during suspension prior to plating (Figure 5C and 5D, "S"), or during both phases (Figure 5C and 5D, "P+S"). H2009 cells showed a dramatic decrease in clonogenic survival with ERK inhibition regardless of when PD98059 was included (Figure 5C). For A549 cells inclusion of PD98059 also inhibited colony formation, albeit to a lesser extent, and only when included during suspension (Figure 5D). Treatment of H2009 δ scr and δ 193 cells with PD98059 during suspension and plating also inhibited clonogenic growth (Figure 5E), however PD98059 had no significant effect on the clonogenic growth of A549 δ scr or δ 193 cells (Figure 5F).

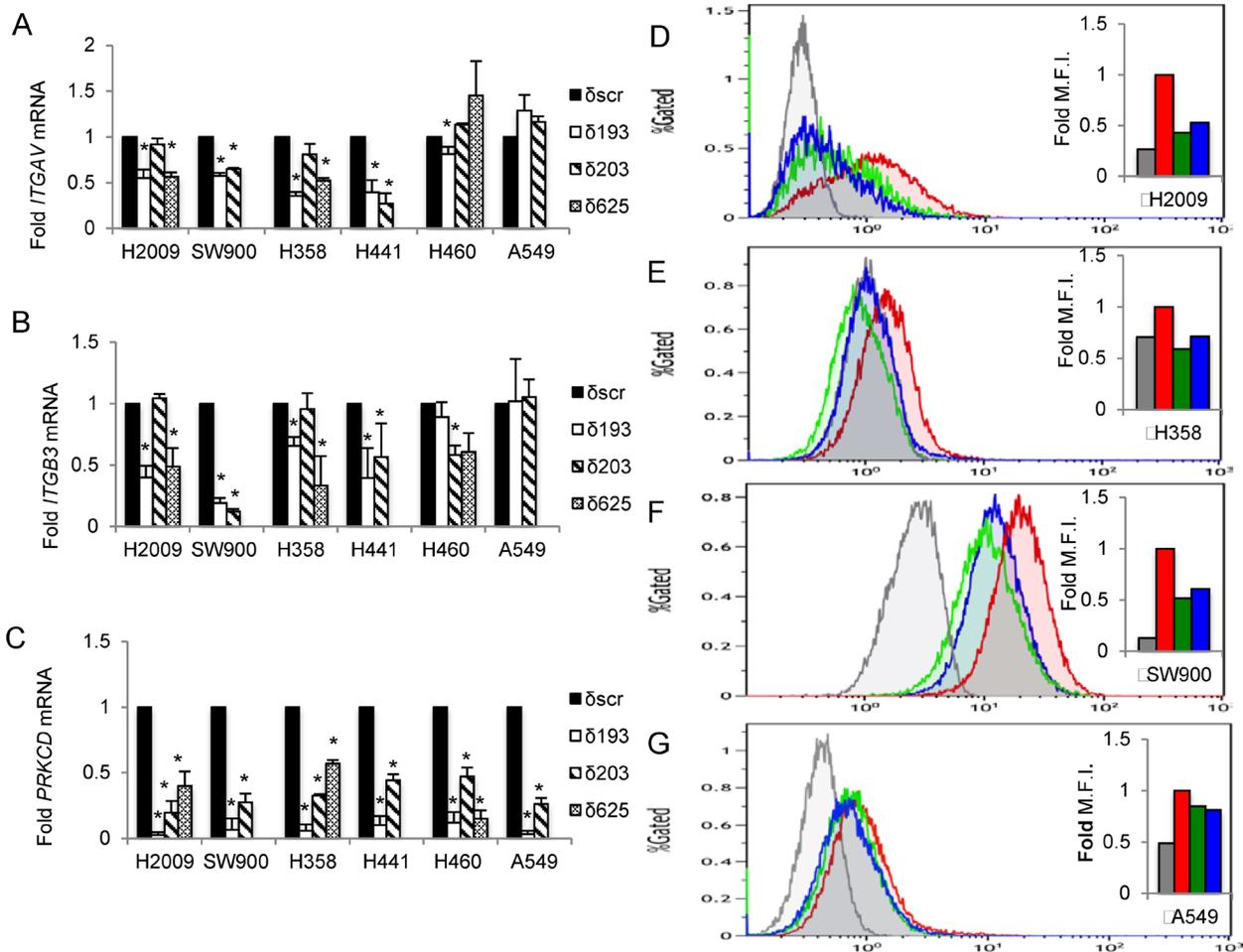


Figure 2: PKC δ regulates *ITGAV* and *ITGB3* mRNA expression in K-ras dependent NSCLC cell lines. PKC δ was depleted in K-ras dependent cells (H2009, SW900, H358, H441) or K-ras independent cells (H460, A549) using unique shRNAs (δ 193, δ 203, δ 625) or a scrambled control shRNA (δ scr) as described in Materials and Methods. **A.-C.** qRT-PCR values of *ITGAV* (A), *ITGB3* (B) and *PRKCD* (C) mRNA expressed as fold of δ scr control; black bars = δ scr, white bars = δ 193, diagonal lined bars = δ 203, and hashed bars = δ 625 shRNA. Error bars are standard error of the mean of three or more experiments, * p < 0.05, Student's t -test. **D.-G.** NSCLC cells expressing shRNA to PKC δ (δ 193, δ 203) or δ scr were analyzed for integrin $\alpha_v\beta_3$ expression at the cell surface by flow cytometry with an antibody that recognizes the integrin $\alpha_v\beta_3$ heterodimer (LM609). K-ras dependent NSCLC cells H2009 (D), H358 (E), and SW900 (F), and K-ras independent cells A549 (G). Inset shows histogram of median fluorescent intensity, red = δ scr, green = δ 193, blue = δ 203, grey = anti-mouse IgG. This experiment was repeated three times; a representative experiment is shown.

PKC δ regulates the transformed growth of K-ras dependent NSCLC cells through an integrin $\alpha_v\beta_3$ and ERK dependent pathway

To determine if reduced ERK signaling contributes to the suppression of transformed growth observed with

PKC δ depletion, we asked if reconstitution of PKC δ could restore ERK activation in H2009 PKC δ depleted cells. As expected, ERK activation was decreased in H2009 $\delta 625$ cells (Figure 6A, left, lane 2) compared to δscr cells (Figure 6A, left, lane 1), however this was completely rescued in cells transduced with Ad-GFP-PKC δ (Figure

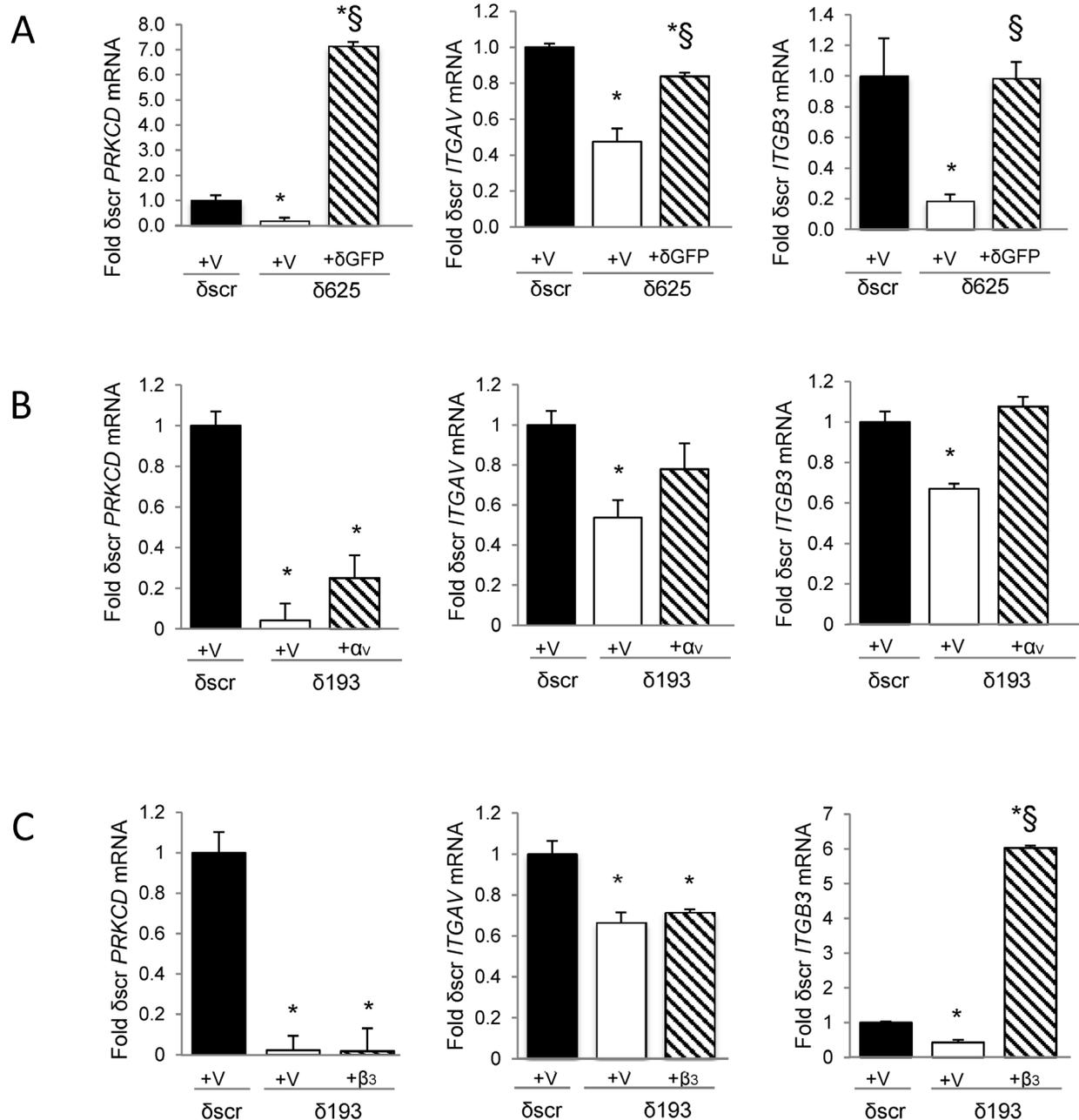


Figure 3: Rescue of PKC δ increases expression of *ITGAV* and *ITGB3* mRNA. **A.** H2009 δscr or $\delta 625$ cells were transduced with Ad-GFP (V) or Ad-GFP-PKC δ (δGFP). **B.** H2009 δscr or $\delta 193$ cells were transiently transfected with pLenti-siLuc (V) or pLenti-*ITGAV* (α_v). **C.** H2009 δscr or $\delta 193$ cells were transiently transfected with pBABE (V) or pBABE-*ITGB3* (β_3). *PRKCD* (left), *ITGAV* (middle), or *ITGB3* (right) mRNA was assayed as described in Materials and Methods and is expressed as fold of δscr control. For panels A, B and C, black bars = δscr + indicated vector, white bars = $\delta 625$ (A) or $\delta 193$ (B, C) + indicated vector, and diagonal lined bars = $\delta 625$ + δGFP (A), $\delta 193$ + α_v (B), or $\delta 193$ + β_3 (C). Values shown are an average of three experiments; error bars are standard error of the mean; * $p < 0.05$ compared to δscr ; § $p < 0.05$ compared to $\delta 193$ or $\delta 625$ (Student's *t*-test).

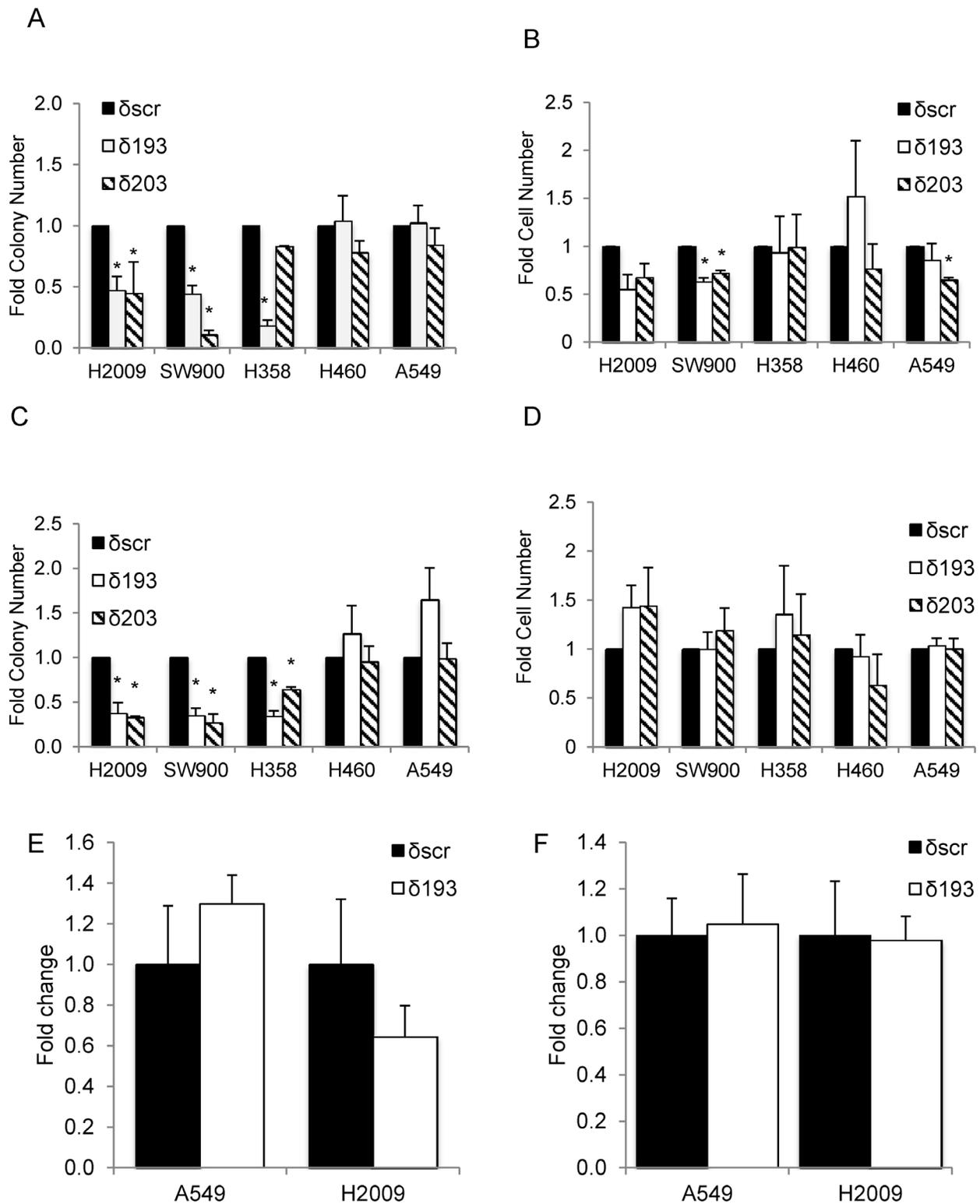


Figure 4: PKC δ regulates survival in K-ras dependent cell lines. K-ras dependent (H2009, SW900, H358) or K-ras independent (H460, A549) δ scr, δ 193 or δ 203 cells were cultured on plastic (adherent culture, panels A., B. and E.) or poly-HEMA coated plates (suspension culture, panels C., D. and F.). Panels A and C show clonogenic survival; panels B and D show number of viable cells after 24 hours of culture as assayed by Trypan Blue exclusion. Black bars = δ scr, white bars = δ 193, diagonal lined bars = δ 203 shRNA expressing cells. For panels E and F, apoptotic cells in adherent (E) or suspension (F) cultures were assayed using a Yo-Pro assay as described in Materials and Methods. Data is shown as fold of δ scr control; black bars = δ scr, white bars = δ 193. Values shown are an average of three experiments, error bars are standard error of the mean, * $p < 0.05$ as compared to δ scr.

Table 2: KEGG Pathways regulated by PKC δ in K-ras dependent and independent NSCLC cell lines

KEGG Pathway	Gene number	Genes	p-value
K-ras Dependent			
Focal adhesion	9	Up: AKT3, RELN, TNC, ZYX	0.006
		Down: ITGAV, ITGA3, ITGB3, MET, TNXB	
ECM-receptor interaction	8	Up: RELN, TNC,	0.0001
		Down: AGRN, DAG1, ITGAV, ITGA3, ITGB3, TNXB	
Chronic myeloid leukemia	5	Up: AKT3, CBLB, MYC	0.019
		Down: NRAS, TP53	
Small cell lung cancer	5	Up: AKT3, MYC	0.028
		Down: ITGAV, ITGA3, TP53	
Sphingolipid metabolism	4	Up: -	0.016
		Down: ASAH1, GALC, NEU1, SGMS2	
Endometrial cancer	4	Up: AKT3, MYC	0.034
		Down: NRAS, TP53	
K-ras Independent			
Pathways in cancer	7	Up: FOS, MDM2	0.041
		Down: CDK6, ERBB2, JAK1, NFKBIA, NRAS	
Prostate cancer	5	Up: CREB3, MDM2	0.005
		Down: ERBB2, NFKBIA, NRAS	
Jak-STAT signaling	5	Up: IL20RB, SPRY4	0.032
		Down: IL6ST, JAK1, LIFR	
Chronic myeloid leukemia	4	Up: MDM2	0.021
		Down: CDK6, NFKBIA, NRAS	
Toll-like receptor signaling	4	Up: FOS,MAP2K3	0.044
		Down: NFKBIA, TLR3	
Bladder cancer	3	Up: MDM2	0.043
		Down: ERBB2, NRAS	

All significant ($p < 0.05$) KEGG pathways are shown for 210 genes regulated by PKC δ in K-ras dependent (H2009 and H441 cells); 94 genes up regulated and 116 genes down regulated. All significant ($p < 0.05$) KEGG pathways are shown for 124 genes regulated by PKC δ in K-ras independent (A549 and H460 cells), 47 genes up regulated and 77 genes down regulated.

6A left, lane 2 versus lane 3). Similarly, expression of integrin α_v also restored ERK activation in H2009 PKC δ depleted cells (Figure 6A, right, lane 2 versus lane 3). Reconstitution of either PKC δ or integrin α_v expression likewise completely restored transformed growth (Figure 6B). To ask if integrin $\alpha_v\beta_3$ is required for PKC δ regulation of transformed growth, H2009 $\delta 625$ cells were transduced with Ad-GFP-PKC δ or Ad-GFP and transformed growth was assayed in the presence of an integrin $\alpha_v\beta_3$ blocking antibody. Inclusion of the integrin $\alpha_v\beta_3$ blocking antibody completely blocked the increase in transformed growth seen with re-expression of PKC δ , indicating an absolute requirement for $\alpha_v\beta_3$ signaling downstream of PKC δ (Figure 6C). As our data shows that both PKC δ and

integrin $\alpha_v\beta_3$ regulate ERK activation and transformed growth of K-ras dependent NSCLC cells (Figure 6A and 6B), and that integrin $\alpha_v\beta_3$ is a downstream effector of PKC δ in this pathway, we next asked if integrin $\alpha_v\beta_3$ regulation of ERK activation is required for transformed growth. H2009 $\delta 625$ cells were transfected with pLenti-*ITGAV* or a pLenti-siLuc control vector and PD98059 was included during the clonogenic survival assay. As shown in Figure 6D, expression of *ITGAV* increased transformed growth of H2009 $\delta 625$ cells, and this could be nearly completely blocked by PD98059, indicating a requirement for ERK downstream of both PKC δ and integrin $\alpha_v\beta_3$ for survival under anchorage independent conditions. Taken together, our data defines a novel mechanism through

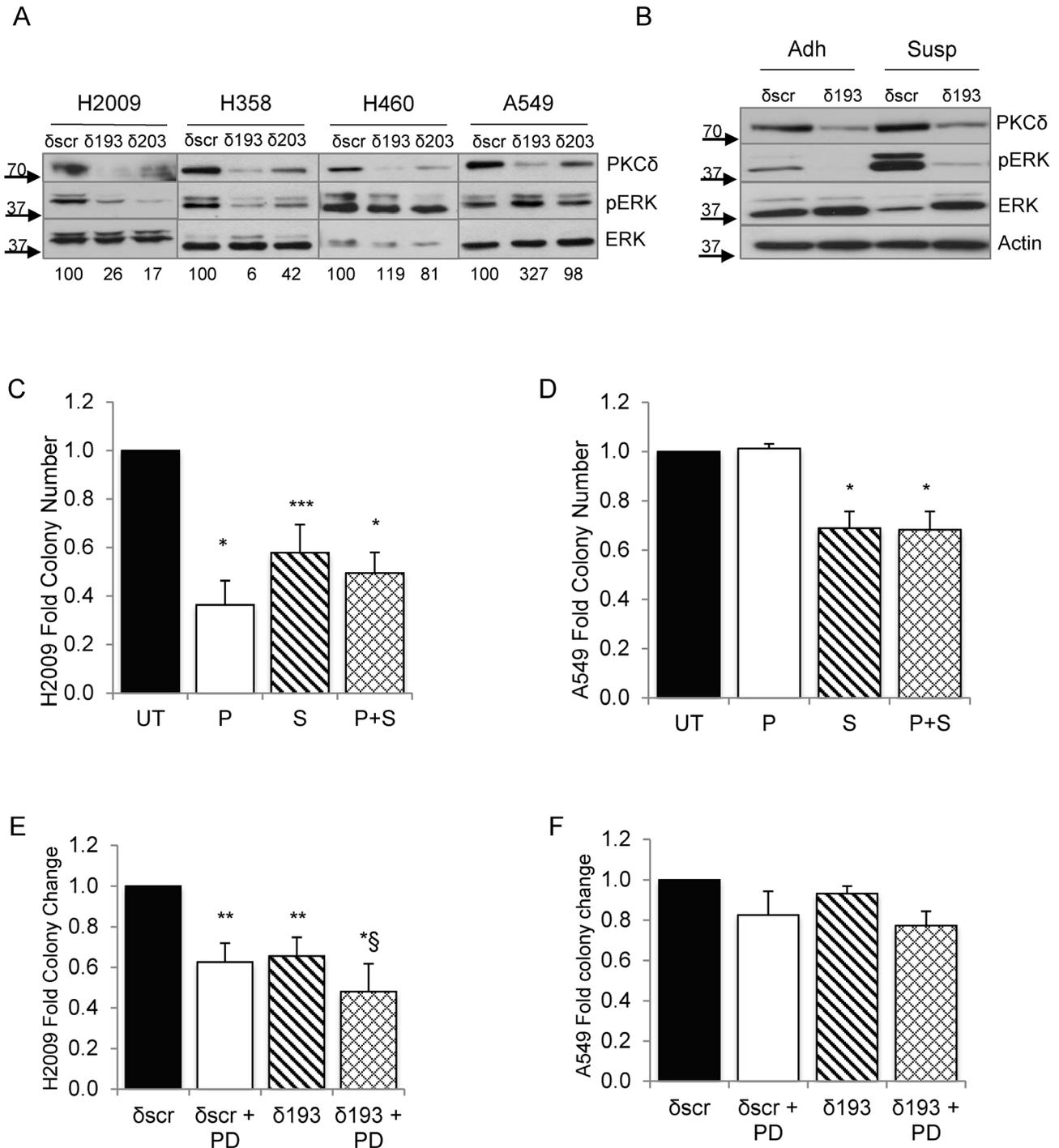


Figure 5: ERK activation is regulated by PKC δ and integrin α_v . **A.** A549, H460, H2009, and H358 δ scr, δ 193 or δ 203 cells were cultured on poly-HEMA coated plates for 24 hrs. Cells were harvested and probed for expression of the indicated proteins by immunoblot; values under blots show fold pERK/ERK for each cell line as determined by densitometry. **B.** H2009 δ scr and δ 193 cells were cultured under adherent conditions (Adh) or on poly-HEMA coated plates (Susp) for 4 hrs prior to harvest. Cells were harvested and probed for expression of the indicated proteins by immunoblot. For panels **C.** and **D.**, H2009 or A549 cells were maintained in suspension for 24 hours prior to plating for a clonogenic assay. Cells were left untreated (“UT”, black bars) or treated with 60 μ M PD90859 as follows: during plating only (“P”, white bars), during suspension culture only (“S”, diagonal lined bars), or during plating and suspension culture (“P+S”, hashed bars). For panels **E.** and **F.**, H2009 δ scr or δ 193 cells (**E.**), or A549 δ scr or δ 193 cells (**F.**) were maintained in suspension culture with or without the addition of 60 μ M PD90859 for 24 hours prior to plating for a clonogenic assay. For both panels, black bars = δ scr, white bars = δ scr + PD90859, diagonal lined bars = δ 193, and hashed lines = δ 193 + PD90859. Values shown are an average of three experiments; error bars are standard error of the mean. * p < 0.05 compared to δ SCR, ** p < 0.08 compared to δ SCR, *** p < 0.10, § p < 0.1 compared to δ 193, Student’s t -test.

which PKC δ regulation of $\alpha_v\beta_3$ expression and ERK activation controls transformed growth of NSCLC cells in the context of K-ras dependency. Of note, our studies also show that *ITGAV* can regulate PKC δ mRNA and protein expression (Figure 3B and 6E), thus transformed growth in this context may be sustained in part through a positive feedback mechanism that assures activation of ERK dependent survival pathways.

DISCUSSION

Oncogenic mutations in *KRAS* are found in about 25% of lung adenocarcinomas, however many tumors with mutations in *KRAS* are no longer “dependent” on K-ras for survival, thus targeting these tumors will depend on understanding the molecular underpinnings of K-ras dependence [3, 4]. Our previous studies have defined PKC δ as a key mediator of K-ras dependent tumorigenesis in NSCLC [11]. In an effort to understand how PKC δ functions in the context of oncogenic *KRAS*, we analyzed PKC δ regulated gene expression in K-ras dependent and independent NSCLC cell lines. Our studies identify the integrin heterodimer, $\alpha_v\beta_3$, as a critical target of PKC δ in the context of K-ras dependency, and define a novel PKC δ \rightarrow integrin $\alpha_v\beta_3$ \rightarrow ERK pathway that regulates the transformed growth of human K-ras dependent NSCLC cells.

Expression profiling of genes regulated by PKC δ reveals changes in the expression of over 3000 genes in *KRAS* mutated NSCLC cells, confirming earlier studies by Caino, *et al.* that defined PKC δ as a major transcriptional regulator of genes induced by phorbol ester [24]. Similar to Caino *et al.*, we found that the majority of these genes were down regulated with depletion of PKC δ , supporting a role for PKC δ in positively regulating their transcription. KEGG pathway analysis suggests that PKC δ can influence a wide variety of cellular pathways and processes, a concept supported by functional data from many labs [25, 26] (Table 1). Two main themes are evident from our analysis. First, PKC δ likely contributes to the regulation of metabolic processes that control nutrient availability and oxidative stress, as depletion of PKC δ increases the expression of genes that drive amino acid, nitrogen and glutathione metabolism. Genes in the pentose phosphate pathway, important for production of NADPH, are also regulated by PKC δ , consistent with known roles for PKC δ in regulation of ROS and NADPH [27, 28]. Second, PKC δ is important for supporting survival signaling, including “outside-in” signaling. This is consistent with the pro-survival properties attributed to PKC δ in tumor models, including known roles for PKC δ in invasion and migration [11, 29-31], and our own data that shows increased PKC δ expression correlates with a worse prognosis in breast cancer patients [6, 12]. Notably, several genes in the *ECM-receptor interaction* and *Focal adhesion* KEGG pathways identified in our studies were also identified as PKC δ

regulated genes by Caino *et al.* including *TNC*, *ZYX*, *MET* and *TNXB* [24].

We show that pro-survival integrin $\alpha_v\beta_3$ is a target of PKC δ in K-ras dependent NSCLC cells, and that re-expression of PKC δ restores expression of *ITGAV* and *ITGB3* mRNA (Figure 3). Our studies suggest that expression of the integrin $\alpha_v\beta_3$ heterodimer is coordinated at the level of transcription or mRNA processing/stability. While others have shown that increased expression of one integrin subunit can stimulate cell surface expression of its binding partner, transcriptional regulation by partner integrins has not previously been reported [32, 33]. Previous reports do however suggest coordinated transcriptional regulation of *ITGAV* and *ITGB3* genes, including a recent study that demonstrates inhibition of *ITGAV* and *ITGB3* transcription by Myc in a breast cancer model [33]. Likewise, thyroid hormone has been shown to regulate transcription of *ITGAV* and *ITGB3* through an ERK dependent process [34]. In our studies re-expression of integrin α_v also increases *PRKCD* mRNA and protein expression, thus we cannot rule out a role for PKC δ in regulation of *ITGB3* mRNA expression.

Our findings identify PKC δ as a potential regulator of tumor progression and metastasis through modulation of integrin $\alpha_v\beta_3$ regulated survival pathways. Notably, PKC isoforms, including PKC δ , have been previously shown to regulate integrin adhesion [29], signaling [35-37], and $\alpha_v\beta_3$ integrin mediated invasion [38]. PKC δ has also been linked to survival signaling through regulation of growth factor receptor and receptor tyrosine kinase activation of MAPK family of signaling cascades [7, 11, 12, 39-43]. Activation of ERK is required for survival of cells in suspension [23], and alterations in ERK activation may contribute to anoikis resistance, a hallmark of metastatic tumor cells [44]. We provide evidence of ERK regulation by PKC δ in K-ras dependent, but not K-ras independent NSCLC cells, which could account for the specific effect of PKC δ depletion on clonogenic survival (Figure 5A). As depletion of PKC δ does not reduce survival in suspension culture or induce apoptosis, ERK activation downstream of PKC δ during suspension may be required to prime cells for survival under clonogenic conditions. PKC δ regulates ERK activation through integrin $\alpha_v\beta_3$, as re-expression of either PKC δ or integrin α_v restores transformed growth and activates ERK (Figure 6A and 6B). Furthermore, we show that restoration of transformed growth in H2009 $\delta 625$ cells reconstituted with PKC δ requires integrin $\alpha_v\beta_3$ (Figure 6C). Our studies however do not exclude a role for PKC δ in regulation of ERK activation upstream of integrin $\alpha_v\beta_3$ or through integrin $\alpha_v\beta_3$ independent mechanisms. In this regard, recent studies by Kurihara *et al.* demonstrate a PKC-ERK- $\alpha_v\beta_3$ pathway that regulates Tumor Necrosis Factor- α production in monocytes [45].

Patients harboring tumors with oncogenic *KRAS* mutations are generally refractive to available therapies, resulting in poor clinical outcomes. Our studies support

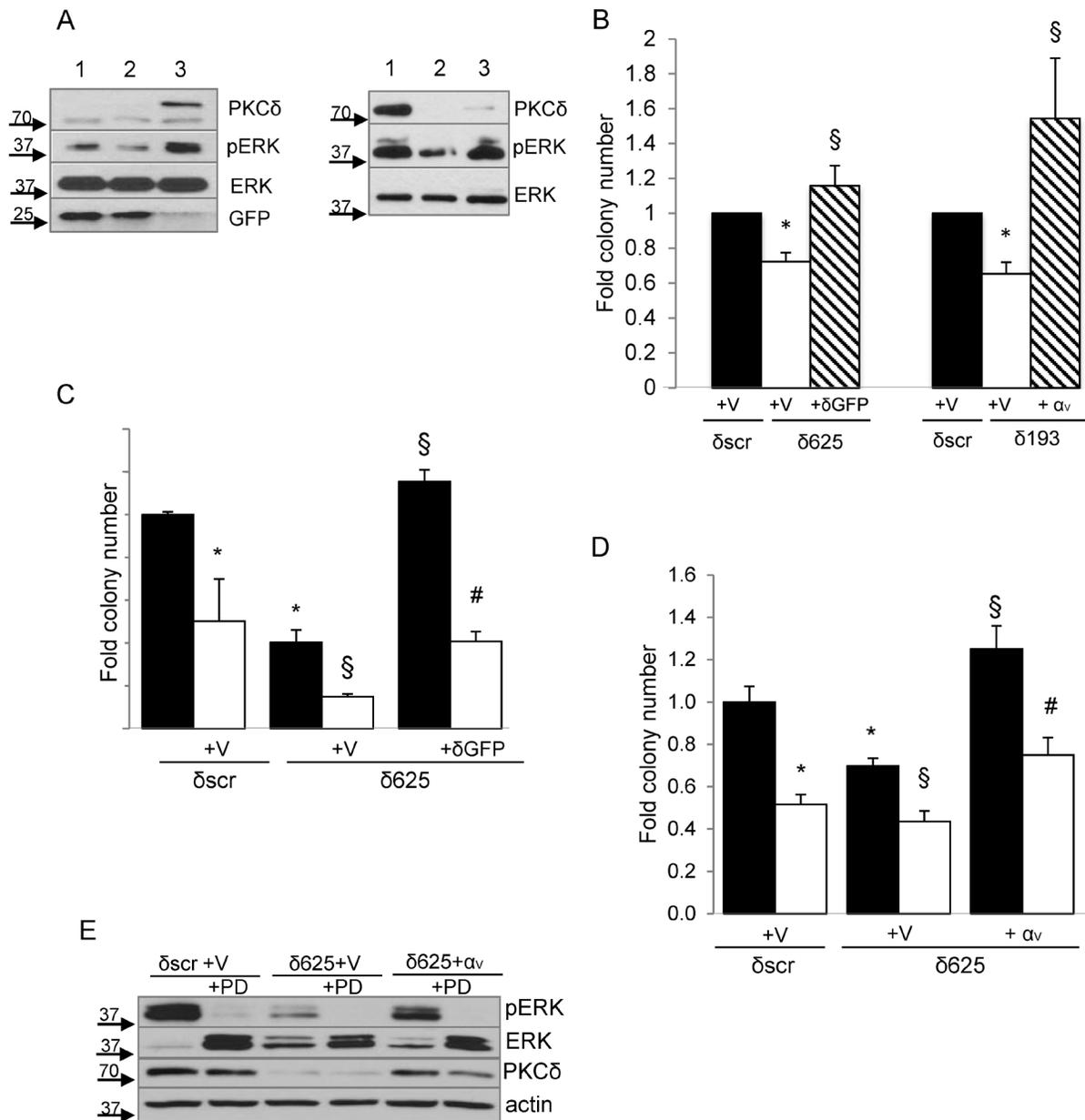


Figure 6: PKC δ regulation of transformed growth requires integrin $\alpha_v\beta_3$. **A.** Left panel, H2009 δ scr cells were transduced with Ad-GFP (lane 1), or H2009 δ 625 cells were transduced with Ad-GFP (lane 2) or Ad-GFP-PKC δ (lane 3). Right panel, H2009- δ scr cells were transiently transfected with pLenti-siLuc (lane 1) or H2009 δ 193 cells were transiently transfected with pLenti-siLuc (lane 2) or pLenti-ITGAV (lane 3). Cells were harvested and probed for expression of the indicated proteins by immunoblot. Arrows indicate the position of endogenous and GFP- PKC δ . **B.** For PKC δ reconstitution, H2009 δ scr (black bars) and δ 625 cells (white bars) were transduced with Ad-GFP (V), or δ 625 cells were transduced with Ad-GFP-PKC δ (δ GFP) (diagonal lined bars) prior to plating in the clonogenic assay. For integrin α_v rescue, H2009 δ scr (black bars) and δ 193 cells (white bars) were transiently transfected with pLenti-siLuc (V), or H2009 δ 193 were transiently transfected pLenti-ITGAV (α_v) (diagonal lined bars) prior to plating in the clonogenic assay. Values shown are an average of six experiments. Error bars represent the standard error of the mean. **C.** H2009 δ scr or δ 625 cells (black bars) were transduced with Ad-GFP (V) or Ad-GFP-PKC δ (δ GFP) and put in suspension culture for 24 hours with the inclusion of 20 μ g/ml of an integrin $\alpha_v\beta_3$ function blocking antibody (LM609) (white bars) or 20 μ g/ml anti-mouse IgG (black bars) prior to plating in a clonogenic assay. This experiment was repeated four times; a representative experiment is shown. Error bars represent the standard error of the mean. **D.** H2009 δ scr or δ 625 cells (black bars) were transfected with pLenti-siLuc (V) or pLenti-ITGAV (α_v) as indicated, and then put in suspension culture for 24 hours without (black bars) or with (white bars) the inclusion of 60 μ M PD98059 prior to plating in a clonogenic assay. Sixty μ M PD98059 was also included in the plating media. This experiment was repeated three times; a representative experiment is shown. Error bars represent the standard error of the mean. For graphs B, C, D: * $p < 0.05$ compared to δ scr, $\S p < 0.05$ compared to δ 193 or δ 625, # $p < 0.05$ compared to δ 625- δ GFP/ α_v . **E.** A representative immunoblot of cells transfected in (D) and probed for expression of the indicated proteins.

exploration of PKC δ as a drug target in KRAS mutant lung cancer. We show that PKC δ regulation of integrin $\alpha_v\beta_3$ survival signaling is specific for K-ras dependent NSCLC cells. As K-ras dependent and independent phenotypes have been demonstrated in other human cancers with oncogenic K-ras, including pancreatic adenocarcinoma and colon cancer [4], the pathways defined here may contribute to tumor progression in many types of human cancer. Based on previous studies by the Cheresh lab, which correlate increased integrin $\alpha_v\beta_3$ expression with tumor progression, the PKC δ \rightarrow integrin $\alpha_v\beta_3$ \rightarrow ERK pathway we describe may have consequences for the metastatic potential of cancer cells [18]. PKC δ has been implicated as a pro-metastatic factor in breast cancer models [46] and in many tumor phenotypes associated with metastasis [47] such as migration and invasion [11]. Our studies suggest that inhibition of PKC δ in K-ras dependent NSCLC may decrease the expression of integrin $\alpha_v\beta_3$, a known regulator of cell survival *in vitro*, and a factor associated with metastasis *in vivo* and in patients. Identifying patients most likely to benefit from targeting the PKC δ survival pathway will depend on genetic and/or functional markers of PKC δ dependency. The co-segregation of PKC δ and K-ras dependency will help to focus this effort, while providing new therapeutic options for patients with K-ras dependent cancers.

MATERIALS AND METHODS

NSCLC cell lines and PKC δ depletion

NSCLC cell lines were acquired through the University of Colorado Denver Lung Cancer SPORE cell bank. Cell line profiling for authentication was done at the DNA sequencing Core at University of Colorado Anschutz Medical Campus using the ABI profiler plus and ABI Identifiler profiling kits. NCI-H2009 (H2009), NCI-H358 (H358), SW-900 (SW900), NCI-H441 (H441), NCI-H460 (H460) and A549 cells were cultured in RPMI-1640 with 2 mM L-glutamine and supplemented with 10% fetal bovine serum. Stable depletion of PKC δ was done as previously described using lentiviral constructs containing shRNA to human PKC δ [pLKO-TRC00010193 (δ 193) or pLKO-TRC00010203 (δ 203) from Open Biosystems, or TRCN0000196625 (δ 625) from SigmaAldrich] or a scrambled shRNA control (pLKO-scrambled (δ scr) from Open Biosystems) [11]. Stable cell lines were continuously maintained in 2 μ g/mL puromycin. For PKC δ rescue, H2009 δ 625 or δ scr cells were transduced with an adenovirus that expresses GFP-tagged murine PKC δ (Ad-GFP-PKC δ) or GFP adenovirus (Ad-GFP) at multiplicity of infection (MOI) of 25 or 50. For integrin α_v rescue, H2009 δ 193 or δ scr cells were transfected with pLenti-*ITGAV* or pLenti-siLUC (1.5 ng DNA/5 x 10⁴

cells). For integrin β_3 rescue H2009 δ 193 or δ scr cells were transfected with pBabe-*ITGB3* or control pBabe using Fugene (Promega #E2311) as transfection reagent. In some experiments an integrin $\alpha_v\beta_3$ function blocking antibody (LM609) (Millipore #MAB1976) or anti-mouse IgG (Millipore #CBL610) was included at 20 μ g/ml.

DNA microarray and data analysis

mRNA from three biological replicates of NSCLC cells stably transduced with δ scr or δ 193 shRNA were profiled on Affymetrix Human Gene 1.0 ST gene arrays by the Microarray Core, University of Colorado Denver Anschutz Medical Campus. Raw data were normalized using Affymetrix Power Tools (APT) using Robust Multiarray Average method. Genes whose expression were statistically significantly different in the three δ 193 replicates, (Student's *t*-test, $p < 0.05$), and which showed a 1.25 fold change or greater over the δ scr replicates, were selected for further analysis. Candidate genes were analyzed for enriched pathways using National Institutes of Health Database for Annotation, Visualization, and Integrated Discovery (NIH DAVID) program [48, 49] and the KEGG analysis program [21, 22]. Raw microarray data has been deposited to NCBI Gene Expression Omnibus with the accession number GSE72788.

Quantitative real time polymerase chain reaction (qRT-PCR)

Analysis was completed as previously described [50]. In brief, total RNA was purified from cells using RNeasy mini kits (Qiagen) and were reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Thermo #EP0741). The reverse transcription reactions were analyzed by PCR using Absolute Blue qPCR SYBR Green Mix (Thermo #AB-4166) in an iCycler (BioRad) thermal cycler. qRT-PCR analysis of mRNA was carried out with forward and reverse primers as indicated (Supplementary data, Table S3). Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression as measured by qRT-PCR in replicate samples. Data are presented as "relative expression," or "fold δ scr mRNA."

Clonogenic survival assay and suspension culture

Tissue culture plates were coated with 1% poly (2-hydroxyethyl methacrylate) (polyHEMA; Sigma P3932) solution in 95% ethanol, and allowed to completely dry at room temperature. Cells (1 x 10⁵ cells/mL) were cultured for 24 hours on 1% polyHEMA plates to prevent adhesion, collected, washed with PBS and 2 mM EDTA and replated into 6-well plates (100 - 1000

cells/well). For some experiments ERK signaling was inhibited by pre-treating NSCLC cell lines with 60 μ M PD98059 for 45 minutes prior to plating. In some cases cells were maintained in PD98059 for the duration of the clonogenic assay. Colonies were stained with crystal violet (0.5% crystal violet, 6% glutaraldehyde), and quantified using ImageJ [20, 51].

Flow cytometry

Cells were cultured under normal adherent conditions for 48 hours prior to analysis, then lifted with Ethylenediaminetetraacetic acid (EDTA) and washed three times in phosphate-buffered saline (PBS). Non-specific binding was blocked with goat serum, and cells were incubated with primary antibody to integrin $\alpha_v\beta_3$ (LM609) (Millipore #MAB1976) or anti-mouse IgG, negative control (Millipore #CBL610). Cells were washed in PBS with 0.5% bovine serum albumin and 0.1% sodium azide and incubated with phycoerythrin-conjugated secondary antibody (BD#550589). Median fluorescence was measured by the University of Colorado Anschutz Medical Campus Flow Cytometry Core on Gallios flow cytometry machine and analyzed on Kaluza (Beckman Coulter). To assay apoptosis, A549 or H2009 δ scr or δ 193 cells were plated at a concentration of 1×10^5 cells/ml on either 1% polyhema coated plates (suspended) cells or on regular tissue culture plates (attached) for 24 hours. Cells were harvested and stained for flow cytometry using TrypLE express dissociation reagent containing a mixture of 0.1 μ M Yo-Pro and 8.1 μ M Hoechst 33342 stain (Chromatin Condensation/Membrane Permeability/Dead Cell Apoptosis Kit with Hoechst 33342/YO-PRO®-1 and PI for Flow Cytometry, Invitrogen, Catalog #V23201). Cells were then washed with PBS and analyzed by flow cytometry as described above.

Immunoblot analysis

Immunoblotting was performed as previously described [7]. Antibodies to phosphorylated ERK1/2 (#9101) and ERK1/2 (#4695) were purchased from Cell Signaling Technologies. The anti-PKC δ antibody was purchased from Santa Cruz (sc-937); anti-actin-HRP and anti-GFP were purchased from Abcam (ab49900 and ab290, respectively).

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest.

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Editorial note

This paper has been accepted based in part on peer-review conducted by another journal and the authors' response and revisions as well as expedited peer-review in Oncotarget.

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